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ADVANCES IN ENZYMOLOGY

**AND RELATED SUBJECTS OF
BIOCHEMISTRY**

Volume VIII

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ADVANCES IN ENZYMOLOGY

AND RELATED SUBJECTS OF BIOCHEMISTRY

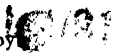
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ADVANCES IN ENZYMOLOGY

**AND RELATED SUBJECTS OF
BIOCHEMISTRY**

Volume VIII

FUNCTIONING OF THE CYTOPLASM

By LUDWIK MONNÉ, *Stockholm, Sweden*

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I. Introduction

In this article a critical and concise review of the recent literature dealing with the structure and the function of the protoplasm is presented. Moreover, an attempt is made to synthesize our knowledge and to give a consistent picture of the functioning of the cytoplasm. At the present moment this task does not appear to be too daring because of the great progress made during the last ten years in the field of chemistry and submicroscopic morphology of the protoplasm. A general survey of this field may be of some value for further investigations, even if the gaps in our knowledge must be filled by some hypotheses. At any rate, there will be a clear distinction between well-established facts and theories.

Structure and function of the protoplasm are intimately corre-

lated with each other. In biology, structure and function are two concepts similar to matter and energy in physics; they are merely two different aspects of the same thing. Any structure is the expression of the function of the protoplasm. Intimate collaboration between morphologists and physiologists is necessary in order to elucidate the life phenomena of the protoplasm. This collaboration has yielded the most splendid results in the study of heredity, but it is still very feeble in investigations dealing with the life phenomena of the cytoplasm, particularly with the mechanics of embryological differentiation. This article is therefore devoted to the regular, microscopic, and submicroscopic structural changes underlying any function of the cytoplasm.

II. Substrata of the Basic Life Phenomena

Anabolism, catabolism, and irritability are the three basic life phenomena. Growth, reproduction, and development are the expressions of the anabolic activity of the living substance. Irritability is the faculty of the purposeful regulation of both the anabolic and catabolic activities of the protoplasm.

Teleological thinking is required in order to describe life phenomena adequately. Causal thinking is necessary in order to find a scientific explanation of the indubitable purposefulness in nature (56). This purposefulness cannot be explained by assuming the existence of a mystic agent. It must be sought in the organization of the living substance. The power of reacting purposefully persists even when the organization of the protoplasm is disturbed to a minor degree. If, however, this organization is severely disturbed, nonpurposeful and detrimental processes appear. This is the case in many pathological conditions. Life phenomena are purposeful because they are always the result of a compromise among a great variety of antagonistic, stimulating, and inhibiting agents: *pH* buffering is the simplest inanimate system exhibiting a certain "purposefulness" (56).

The following units of life should be distinguished:

- I. Ultimate units of life (viruses, genes, microsomes, etc.)
- II. Cells.
 1. Prokaryote cells (bacteria, *Cyanophyceae*).
 2. Eukaryote cells (cells of all other organisms).
- III. Multicellular organisms.
- IV. Colonies of multicellular organisms.

Nucleic acid is concerned with the anabolic activity of the protoplasm, particularly with protein synthesis (see page 58). This is evidenced by the following facts: The amount of nucleic acid is increased in all kinds of cells when growth is initiated (13,19). Nucleic acid is not diffusely distributed; it is present within small self-perpetuating bodies (chromomeres, chromidia = microsomes, macromolecules or particulates), which are the most essential constituents of the protoplasm. Moreover, the simplest viruses are pure nucleoproteins (148) that are able to grow and to reproduce themselves within host cells. The catabolic activity of the protoplasm is due to the enzymes of hydrolysis, respiration, fermentation, phosphorylation, etc. Lipides* are concerned with the irritability of the protoplasm (page 42).

Since the simplest viruses are pure nucleoproteins, they exhibit anabolic activity only. They contain no enzymes or lipides and therefore do not exhibit catabolism and irritability. The host cells provide the energy and the material necessary for the anabolic activity of these viruses. There exist all intermediary forms between viruses and bacteria. The highest viruses, such as vaccinia virus, contain some enzymes and lipides (148) and therefore may be endowed not only with anabolic activity but also with a certain catabolism and irritability. At any rate, anabolism, catabolism, and most probably, irritability are displayed by the chromidia (microsomes) extracted from numerous cells.

The simplest protoplasm is represented by viruses, which are, as stated above, pure nucleoproteins. The protoplasm of vaccinia virus has a slightly higher organization, and that of prokaryote and eukaryote cells an even higher organization. It has long been supposed that cells are composed of still smaller units exhibiting all the essential features of life (Hermann, Pflüger, Verworn, Altmann *et al.*; see 58 and 61). This is confirmed by recent investigations (for literature, see 13 and 42). Viruses should be regarded, at least in a restricted sense, as ultimate units of life. But cells cannot be regarded as ultimate units of life. The protoplasm of prokaryote and eukaryote cells is an organized system of several types of self-perpetuating nucleic-acid-containing bodies comparable to viruses. These bodies, however, are not free, but concatenated with each other into self-

* Phosphatides and cholesterol are chiefly in question here.

perpetuating fibrils (cytoplasmic fibrils, chromosomes, etc.). Consequently the cells must be regarded as organized systems of several kinds of self-perpetuating fibrillar components. The higher the organization of the living substance, the more differentiated are the life phenomena. The simplest are exhibited by the primitive viruses, the most complicated by multicellular organisms.

Eukaryote cells have a higher organization than prokaryote cells. The former have a well-developed nucleus that forms chromosomes during mitosis. The latter contain a primitive nucleus (for literature, see 37), very different from the nucleus of eukaryote cells.

III. Structure of Cytoplasm

A. CHROMIDIA AND CYTOPLASMIC FIBRILS

In the first decennium of this century much effort was spent in order to prove that the chromatin regularly migrates from the nucleus into the cytoplasm. Hertwig denominated as chromidia all the cytoplasmic substances supposed to originate directly from the nuclear chromatin. The chromidial hypothesis was developed chiefly by Goldschmidt. For details and literature concerning this subject the reader is referred to Cowdry's book (30). It has been assumed that both the chromatin and the chromidia contain nucleic acid. Moreover, it has been demonstrated by Van Herwerden (see Brachet, 13) that the chromidia in preparations previously exposed to the action of enzymes which break down nucleic acid can no longer be stained with pyronine. Not only minute granules scattered throughout the whole cytoplasm, but also large inclusions of various shapes have been described as chromidia. Unfortunately only a few investigators have been able to distinguish chromidia from mitochondria. The two components of the cytoplasm have been confused by most cytologists, who, as a result, have denied the real existence of the chromidia.

There is no evidence that the chromidia originate directly from the nuclear chromatin; therefore this opinion cannot be accepted by modern cytologists. The general idea, however, that certain cytoplasmic granules are similar to, although not identical with, chromatin is correct and is strongly supported by modern investigations. Moreover, the presence of nucleic acid within the chromidia should be regarded as definitely proved. Nevertheless, an important difference between the chromidia and the chromatin escaped the

previous workers in cytology, because no methods were available to distinguish ribo- from thymonucleic acid in microscopic preparations. These methods have been provided by the independent investigations of Brachet, Caspersson, and co-workers (for literature, see 13,42). It has been demonstrated by Caspersson and collaborators (Schultz, Hydén, Landström-Hydén, Aquilonius, *et al.*) that the cytoplasm of various rapidly growing cells is Feulgen negative and that it strongly absorbs ultraviolet light of the wave length 2600 Å; both properties are characteristic of pure ribonucleic acid. Moreover, it has been found that the chromosomes also strongly absorb the same ultraviolet light and that they are, in contrast to the cytoplasm, Feulgen positive; these properties are characteristic for pure thymonucleic acid. From these facts it has been concluded that ribonucleic acid is present within the cytoplasm and thymonucleic acid within the chromosomes. Another method has been invented by Brachet (13), who found that the cytoplasm of various cells is deeply stained with pyronine when the microscopic preparations are treated with Unnas dye mixture and that the staining fails to occur when the preparations are subjected to the action of the enzyme ribonuclease before treatment with the above-mentioned dye mixture. Thus, it is evident that staining with pyronine is due to ribonucleic acid and that it does not occur when this acid is removed under the influence of the above-mentioned enzyme. Ribonucleic acid is not diffusely distributed throughout the cytoplasm; it is present within minute granules only, long since described as chromidia. Only these granules absorb ultraviolet light, stain with pyronine, and are Feulgen negative.

Recent investigations definitely prove that the chromidia and the mitochondria are entirely different from each other. This is clearly evidenced by the fact that, in sea urchin eggs stratified by centrifuging, the chromidia and the mitochondria are found in two different layers. Only the chromidial layer stains distinctly with pyronine (Monné 98,100) and strongly absorbs ultraviolet light of 2600 Å wave length (57). In contrast to the chromidia, the mitochondrial layer does not stain with pyronine and does not absorb ultraviolet light of the mentioned wave length. It has also been found that the mitochondria of some other cells do not absorb ultraviolet light to any considerable degree (78). Thus, the mitochondria, in contrast to the chromidia, do not contain any considerable amount of ribo-

nucleic acid. Both mitochondria and chromidia are Feulgen negative.

The term chromidia should be restricted to the minute granules only, which exhibit the above-mentioned properties. Nevertheless, the same properties are also exhibited by several other, large structural entities which have also been described as chromidia. These large components of the cytoplasm have the form of thick cords, spirals, concentric lamellar bodies, etc., and do not correspond to the single chromidia. They are compact aggregates of numerous chromidia, present within strongly condensed regions of the fibrillar ground cytoplasm. The known term ergastoplasm should be reserved for all these condensations of the cytoplasmic texture. Like chromatin, the chromidia are purely morphologic entities; their chemical composition is variable. Both the amount of ribonucleic acid present within the chromidia and the amount of thymonucleic acid present within the chromatin granules are subject to great variations under different physiologic conditions. The ribonucleic acid content of the chromidia is high in rapidly growing cells and low in cells that have ceased to grow. In the former case, the chromidia have a more acid character and therefore they are preferentially stainable with basic dyes. In the latter case the chromidia have a more basic character and therefore they are preferentially stainable with acid dyes.

In preparations fixed in Bouin fluid and stained with iron hematoxylin or other basic dyes, the chromidia of sea urchin eggs appear as minute granules whose size is just on the limit of microscopic resolution. Thus, the diameter of the fixed and stained chromidia cannot exceed $0.2\ \mu$. Under the influence of acetic acid contained in Bouin fixative the chromidia swell, similarly to the chromatin granules. The chromidia also increase in size under the influence of the dye, which is heavily adsorbed on their surfaces. Thus the chromidia must be much smaller in living cells, probably $0.1\ \mu$ or less. The chromidia are, as a rule, not densely packed within the cytoplasm and therefore they clearly appear as single bodies in fixed preparations.

The microsomes, macromolecules, plasmagenes, or particulates separated from various minced cells by Claude, Stern, Brachet, Jeener, *et al.* (for literature, see 13,42) contain ribonucleic acid and have a diameter of 0.3 to $0.05\ \mu$. There is no doubt that these corpuscles are identical with the chromidia previously discovered in

fixed preparations. Therefore it is proposed in this article to call these corpuscles chromidia instead of microsomes, etc. The term chromidium is excellent because it means that the corpuscle in question is similar to but not identical with chromatin. Moreover, proper credit should be given to those investigators who first recognized that chromatin-like, nucleic-acid-containing bodies are present within the cytoplasm, and to the man who first coined a suitable term (R. Hertwig) to designate these bodies. The terms microsomes, macromolecules, plasmagenes, or particulates are either too vague or incorrect. Nevertheless, the separation of the chromidia from minced cells is a very important discovery because it renders possible the investigation of the above-mentioned bodies by exact chemical methods.

It is universally accepted that nucleic acid is concerned with protein synthesis (13,19), and that consequently self-perpetuating corpuscles must be nucleoproteins. Thus, it is very probable that the chromidia are self-perpetuating bodies similar to chromatin. The chromidia are an organized system of numerous chemical compounds. The chromidia contain proteins rich in sulfhydryl groups, ribonucleic acid, lipides (among them phosphatides), and respiratory and hydrolyzing enzymes (for details, see page 31; for literature, see 13,42). It is possible that among other proteins the chromidia also contain basic proteins such as histones. In microincinerated preparations heavy ash deposits occur wherever ribo- or thymonucleic acid is present (31,40). Calcium and magnesium are the chief components of this ash. During the development of nerve cells the amount of the ash-yielding substances is strongly decreased within the nuclei and increased within the cytoplasm (76). Simultaneously the amount of nucleic acid (thymonucleic acid) is also strongly decreased within the nuclei and increased (ribonucleic acid) within the cytoplasm. It is evidenced by this fact how intimately nucleic acids are associated with calcium and magnesium. The amount of calcium and magnesium is increased wherever the amount of nucleic acids is increased, and inversely. Moreover, it is known that calcium and magnesium are not free, but bound to the components of the protoplasm (143). Thus, it appears very probable that the phosphoric acid parts of phosphatides and nucleic acids are linked by means of the bivalent cations calcium and magnesium. At any rate, there is no doubt that calcium and magnesium are present

within the chromidia. The role of the lipides is to separate the nucleoproteins from the numerous enzymes present within the chromidia. The chromidia are not scattered in a disorderly manner within the cytoplasm, but concatenated with each other into long fibrils.

Many theories concerning the structure of the protoplasm have been advanced. It is not the right occasion to discuss all these theories here, inasmuch as this problem appears to be already solved. For literature concerning this matter the reader is referred to the books of Henneguy (60) and Seifriz (144). In the present review only some recent investigations definitely proving the fibrous structure of the protoplasm are reported. Polarization optics proved to be a very valuable method for investigating the submicroscopic structure of living and fixed cells. For literature concerning this subject the reader is referred to the books and papers of Schmidt (138,140).

The fibrillar structure of the ground cytoplasm of living and fixed cells can be safely demonstrated by means of the polarization microscope. Various birefringent inclusions of microscopic size scattered throughout the cytoplasm may conceal and simulate the birefringence of the ground cytoplasm. Therefore living uninjured eggs of sea urchins (98,108*) and frogs (115) were stratified by centrifuging, and investigated between crossed nicols in a polarization microscope. A distinct birefringence could be detected within the clear layer of ground cytoplasm deprived of all inclusions. In sea urchin eggs long streaks of variable thickness negatively birefringent in longitudinal direction have been observed within this layer (98). No doubt the fibrillar components of the ground cytoplasm were oriented parallel to each other by centrifuging. The eggs do not suffer any injury when exposed to this experimental treatment. This is evidenced by the well-known fact that centrifuged eggs cleave upon fertilization. In normal eggs the fibrils are oriented in any direction and therefore the ground cytoplasm appears isotropic. Under the influence of centrifuging the fibrils are oriented in a certain direction and therefore the ground cytoplasm becomes birefringent. This birefringence may be increased by weakly hypertonic sea water (98). The described facts prove that the ground cytoplasm of normal living cells has a fibrillar structure.

* Unfortunately it is very difficult for the reader to imagine what kind of optical phenomena have been observed by Moore and Miller (108). The very short note does not seem to be sufficiently clear.

Runnström (122,123) has demonstrated that under the influence of hypertonic sea water great, but completely reversible, structural alterations are produced within the cytoplasm of the sea urchin *Psammechinus miliaris*. Monné (100,103) investigated in detail these alterations by means of the polarization microscope and gave the correct interpretation of the phenomena observed. It was found that under the influence of hypertonic sea water the fibrillar texture of the ground cytoplasm is condensed in the central region of the eggs. Simultaneously a liquid (enchylema) is released and all inclusions of microscopic size (yolk, mitochondria) are forced out from the interstices between the cytoplasmic fibrils. These inclusions form a layer beneath the cortex that is only $1\ \mu$ thick. The whole ground cytoplasm condensed in the central region of the egg presents a polarization cross which is positive in radial direction. In some cases the described alterations do not occur, but the whole egg becomes positively birefringent in radial direction (94,98).

Under the influence of undiluted glycerol either a mosaic of birefringent structures (122,123) appears, or the whole egg becomes positively birefringent in radial direction (126). The whole egg becomes negatively birefringent in radial direction when fixed in absolute alcohol (98,140). Evidence has been provided (98, 100) that this birefringence is due to the submicroscopic structure of the ground cytoplasm, and not to the birefringence and orientation of the microscopic inclusions of the egg.

Fixed eggs treated with absolute alcohol are negatively birefringent in radial direction because the polypeptide chains of the ground cytoplasm are preferably oriented in tangential direction. This birefringence cannot be due to ribonucleic acid associated with the proteins of the cytoplasm, because the hydrophilic nucleic acid molecules can only be oriented parallel (140) and not perpendicular to the polypeptide chains of the proteins, and because proteins and nucleic acids have an intrinsic birefringence of opposite sign. In eggs treated with hypertonic sea water the whole ground cytoplasm is positively birefringent in a radial direction. Nevertheless, even in this case the birefringence is not due to nucleic acid, but to the rod-shaped lipid molecules oriented in radial direction. This is plainly evidenced by the fact that this birefringence is reversed under the influence of lipid-dissolving, but protein- and nucleic-acid-preserving agents. The cytoplasm of pancreas and nerve cells is known to be particularly rich in ribonucleic acid (20,100). Nevertheless, even in this case the birefringence phenomena (100,120) displayed by the cytoplasm cannot be due to ribonucleic acid (100). Either the amount of ribonucleic acid present within the cytoplasmic fibrils is not sufficient to reverse the sign of birefringence of the proteins, or the ribonucleic acid molecules are arranged in a disorderly manner because

they adhere to the sections of cytoplasmic fibrils which are permanently folded. The latter assumption is strongly supported by the facts cited below (page 14). Thus, it is certain that the birefringence phenomena of the ground cytoplasm are due to proteins and to lipides only. The rod-shaped lipid molecules have one hydrophilic and one hydrophobic pole and therefore they must be oriented perpendicular to the hydrophilic polypeptide chains of the proteins (46,140). The described birefringence phenomena make it evident that the ground cytoplasm has a fibrillar structure. The birefringence phenomena, due to lipides and to proteins, are of opposite sign and therefore they strongly compensate each other. In living sea urchin eggs the birefringence, which is due to the lipid component of the cytoplasmic fibrils, appears distinctly when these fibrils are arranged in a certain order under the influence of some experimental agent. The birefringence due to lipides is increased when the polypeptide chains are stretched under the influence of dehydrating agents. No doubt the ordered arrangement of the lipid molecules is increased when this occurs. This birefringence is reversed when the lipides are dissolved. The birefringence due to the proteins of the cytoplasmic fibrils appears and is still more increased when the polypeptide chains are stretched to the maximum upon complete dehydration.

Not only in the mentioned cells, but in practically any cell, birefringence of the ground cytoplasm may appear under the influence of various agents which cause orientation and stretching of the cytoplasmic fibrils, without serious injury (94,95,97). Detailed investigations have been performed in order to distinguish the birefringence of the ground cytoplasm from the birefringence of its inclusions (94, 97). Also under normal physiologic conditions orientation of the cytoplasmic fibrils and temporary birefringence of the ground cytoplasm may appear (94,140).

The chromidia are a component of the ground cytoplasm. This is evidenced by the fact that in eggs stratified by centrifugal treatment the chromidia are always strongly condensed in the layer which corresponds to the ground cytoplasm (57,98). Birefringence appears within this layer because the fibrillar components of the ground cytoplasm are also strongly condensed here under the influence of centrifugal treatment. The chromidia cannot be separated from the cytoplasmic fibrils by means of centrifugal treatment. The chromidia

are an essential component of these fibrils. It can be seen in fixed preparations that the chromidia are concatenated with each other by means of very thin threads which do not show any distinct affinity to any dye. The intermediate threads do not exhibit the characteristic properties of ribonucleic acid. Thus, it must be concluded that the cytoplasmic fibrils are constructed of the ribonucleic-acid-containing chromidia and the ribonucleic-acid-free interchromidia, regularly alternating with each other.

Ample evidence in support of this conclusion is provided by the following experiment (103,104): Mature unfertilized eggs of the sea urchin *Psammechinus miliaris* were exposed for several hours to the action of a 0.2 *N* sodium azide solution in sea water. Cytolysis does not occur but the structure of the cytoplasm is slowly and gradually altered. The extremely thin cytoplasmic fibrils are oriented parallel to each other. Bundles of cytoplasmic fibrils are formed. The bundles gradually become thicker because of the increasing number of cytoplasmic fibrils which join each other. All intermediate stages between single cytoplasmic fibrils and thick bundles are found. The bundles are cross striated (Fig. 1). The thickest bundles contain several hundred, perhaps even one thousand, cytoplasmic fibrils (see page 13). The fact should be stressed, however, that the chromidia conjugate only with the chromidia, and the interchromidia only with the interchromidia. Evidently, there exists a mutual attraction between the corresponding parts of the cytoplasmic fibrils.* The thick bundles of cytoplasmic fibrils consist of ribonucleic-acid-containing and ribonucleic-acid-free sections regularly alternating with each other. They exhibit a great similarity to the salivary chromosomes of *Drosophila*, which consist of alternating sections, with and without thymonucleic acid.

The parallel orientation of the cytoplasmic fibrils may be a phenomenon related to meiotic chromosome pairing. Identical, or at least a very similar, structure is the prerequisite for this pairing. It is well known that chromosomes of deviating structure do not pair. All cytoplasmic fibrils must have an identical, or at least very similar, structure because they are so easily attached parallel to each other with their corresponding regions. Thus, the cytoplasmic fibrils are, in contrast to the chromosomes, not differentiated along their length. It is evident that all chromidia of a certain cell are identical. The same holds true for the interchromidia.

Under the influence of sodium azide the cytoplasmic fibrils are stretched, a fact which undoubtedly favors their parallel orientation.

* The parallel conjugation of cytoplasmic fibrils may be brought about by a process similar to blood clotting. It has been recently demonstrated that the cross striated fibrin fibrils also adhere to each other with their corresponding regions (57a).

Only the interchromidia are strongly elongated and therefore the distances between the chromidia are increased. Evidently the polypeptide chains of only the interchromidia are unfolded by the action of sodium azide. The stretching of the polypeptide chains is due to dehydration. This is evidenced by the fact that a vacuole is expelled at the ends of the bundles of cytoplasmic fibrils. Under normal conditions the cytoplasmic fibrils are strongly hydrated and the poly-



Fig. 1. Cytoplasmic fibrils arranged in cross-striated bundles under the influence of sodium azide.

peptide chains of their chromidia and interchromidia are folded. It seems that the polypeptide chains of the chromidia are permanently folded, in contrast to the polypeptide chains of the interchromidia, whose folding is subject to variations under different physiologic conditions.

Bundles of cytoplasmic fibrils are approximately as long as the egg diameter (about 90 μ). Thus, it is probable that the single cytoplasmic fibrils also have a similar length in eggs not subjected to any experimental treatment. No doubt the length of cytoplasmic fibrils is variable in various cells and depends upon the diameter of the

latter. It may be that the length of cytoplasmic fibrils is variable under different physiologic conditions and that this may be due to fragmentations and reunions. The cytoplasmic fibrils decrease strongly in length during cleavage because they are cut through in transverse direction simultaneously with any cell division. The length of the fibrillar constituents of the cytoplasm of slime molds has been estimated by Moore at about $10\ \mu$ (107).

The chromidia of the sea urchin eggs are about $100\ m\mu$ thick (see page 6). The interchromidia seem to be thinner. Thus, thickness of the single cytoplasmic fibrils may be estimated to $50\text{--}100\ m\mu$. These thin fibrils are visible in the ordinary microscope only because of their length, which is above the limit of microscopic resolution. The cytoplasmic fibrils are comparable to the protofibrils constituting sperm tails. Ballowitz (2) showed, a long time ago, that sperm tails may be decomposed into several extremely thin fibrils, visible in the ordinary microscope. These protofibrils have recently been investigated by means of the electron microscope and it was found that they are about $50\ m\mu$ thick (57, 134, 137). Sperm tails are bundles of protofibrils whose number and thickness are strikingly constant. This fact indicates that fibrils which are about $50\ m\mu$ thick represent a very important submicroscopic structural unit. These protofibrils may be either constituents of thicker fibrils or may occur independently. Thus, the cytoplasmic fibrils seem to be independent protofibrils. Moore (107) found that the cytoplasm of slime molds may pass without injury through the pores of parchment, which are about $50\ m\mu$ in diameter. He inferred from this fact that the cytoplasm of slime molds must be composed of fibrils which are also about $50\ m\mu$ thick. Thus a structural unit corresponding to protofibrils may also be detected in this case. It would be of interest to know whether there exists yet another intermediary structural unit between the cytoplasmic fibrils, which correspond to the protofibrils, and the single polypeptide chains. At any rate, from muscles, fibrils as thin as $5\text{--}15\ m\mu$ have been separated (54). Thus, they are still thinner than the protofibrils of sperm tails. It is not known how many parallel polypeptide chains the cytoplasmic fibrils may contain. Nevertheless, it may be estimated that the cytoplasmic fibrils are bundles of about 2000 polypeptide chains (104), if it is assumed that the latter are as densely packed as in myofibrils (89). Thus, the ground cytoplasm cannot be regarded as a texture of single polypeptide chains. It has a much coarser structure.

Birefringent substances have been extracted from various tissues which were previously minced. Only in the case of muscles is it certain that these substances originate from the cytoplasm. The birefringent protein myosin is in question here. It has not been proved that the birefringent substances extracted from other cells derive from the cytoplasm. It seems that the birefringence of the materials hitherto separated from the cytoplasm of these cells is entirely due to contamination with thymonucleohistone, a substance

which occurs in the nuclei only (14). At any rate, the extracted chromidia (macromolecules, microsomes, particulates) appear globular in the electron microscope (151) and do not show any flow birefringence (14). This is in perfect harmony with the above-mentioned fact that the birefringence of the cytoplasm is not influenced by ribonucleic acid (see page 10). From this fact it has been inferred that the ribonucleic acid molecules are scattered in a disorderly manner because they intimately adhere to the permanently folded polypeptide chains of the chromidia (100,104).^{*} The ground cytoplasm is a texture of fibrils, the latter consisting of the chromidia and interchromidia which regularly alternate with each other. It must be concluded from the above-mentioned facts that the birefringence phenomena displayed by the ground cytoplasm of living and fixed cells are due to the interchromidia. It is obvious that the birefringent interchromidia are dissolved by the methods employed to extract the isotropic chromidia. In this connection it is of interest to note that the cross-striated muscles may also be fragmented in transverse direction into anisotropic (Bowman discs) and isotropic discs. Agents are known some of which dissolve the former, and some the latter (for literature, see 58). Proteins which are birefringent or which may easily be made birefringent are present in the cytoplasm of any cell. This is clearly evidenced by the birefringence phenomena exhibited by the cytoplasm of living and fixed cells. With the exception of myosins in the case of muscles these proteins have not yet been extracted in pure state.

B. COMPARISON OF VARIOUS LIVING FIBRILS

A longitudinal structural periodicity seems to be the essential feature of all, both inanimate and living, protein fibrils. This periodicity is in some cases above, and in others below, the limits of resolution of the ordinary microscope. Structural periodicity of the cytoplasmic fibrils, the prophase chromosomes, and the cross-striated myofibrils can be investigated easily by means of the ordinary microscope. Structural periodicity of smooth myofibrils, sperm tails, fibrin, and connective tissue fibrils (54,57a,134) appears distinctly only in the electron microscope. All these fibrils exhibit a distinct cross striation. It is very probable that other protoplasmic fibrils, particularly neurofibrils, also have a similar periodic structure.

^{*} Nevertheless the isotropy of the chromidia may also be due to compensation.

This is plainly evidenced by the specific mode of nerve conduction (see page 47). Differences in the folding of the polypeptide chains are probably the essential feature of this structural periodicity (134). In some cases, sections with stretched polypeptide chains may regularly alternate with sections with folded polypeptide chains; in other cases, the two alternating sections may differ from each other only in the degree of folding of their polypeptide chains and, in still other cases, the degree of folding of the polypeptide chains may be the same in both sections which differ from each other only in the relative ease with which their polypeptide chains are unfolded. It is probable that this kind of structure represents a buffering mechanism by which the resistance of protein fibrils against disruption is increased. It is certain that inanimate protein fibrils do not contain enzymes and nucleic acids. Within living fibrils nucleic acids together with the enzymes of the energy-yielding activities and of hydrolysis are present and periodically distributed (see page 33). Nucleic acid and enzymes are probably always associated only with the sections of fibrils whose polypeptide chains exhibit the tendency to remain folded.

The metaphase chromosomes are spirally twisted and strongly contracted; therefore their internal structure is not visible. Nevertheless, the prophase chromosomes are stretched and exhibit a distinct structural periodicity. This kind of structure is displayed most distinctly by the famous salivary chromosomes of dipterans. These chromosomes are bundles of several hundreds of chromonemata and in this regard they are very similar to the bundles of cytoplasmic fibrils described in the preceding section. The single chromonemata are probably as thick as the single cytoplasmic fibrils of sea urchin eggs ($0.1\ \mu$). This is probably the thickness of the univalent chromosomes of the resting nuclei. The chromosomes consist of thymonucleic-acid-containing and thymonucleic-acid-free sections regularly alternating with each other.

Pfeiffer (113,114) and Schmidt (139) demonstrated that the living salivary chromosomes display a very weak birefringence which is negative in longitudinal direction. This birefringence is entirely due to the thymonucleic acid present within the chromomeres. Frey-Wyssling (46) calculated that only a very small percentage (about 3%) of the thymonucleic acid molecules is regularly oriented within the chromosomes. Caspersson (18) arrived at the same conclusion earlier by employing his method of dichroism in ultraviolet light. Evidently the elon-

gated thymonucleic acid molecules are poorly oriented because they intimately adhere to the strongly folded polypeptide chains of the chromomeres.

Also in this respect the chromomeres and the chromidia are very similar to each other. The chromomeres remain unchanged when the salivary chromosomes are stretched. Nevertheless, the distances between the chromomeres are increased, because the interchromomeres are strongly elongated (16,113,114). No doubt, the polypeptide chains of both the chromomeres and the interchromomeres are strongly folded under normal conditions. The polypeptide chains of the interchromomeres only are unfolded when the chromosomes are stretched; the polypeptide chains of the chromomeres remain permanently folded. Thus, yet another similarity between the chromosomes and the cytoplasmic fibrils may be detected. Nevertheless, in contrast to the interchromidia, the interchromomeres remain isotropic, even when strongly stretched (113,114). Under the influence of stretching the polypeptide chains of the interchromomeres are certainly very well oriented, parallel to each other. The isotropy can only be due to compensation, probably brought about by lipid molecules which are oriented perpendicularly to the polypeptide chains. This is strongly supported by the fact that large amounts of lipides are present within the nuclei (152). Unfortunately it is not yet known whether the interchromomeres become positively birefringent in longitudinal direction, when stretched salivary chromosomes are treated with absolute alcohol and similar lipid solvents.

The cytoplasmic fibrils and the chromosomes are very similar to cross-striated myofibrils. Certain important differences, however, exist. The cross-striated myofibrils consist of the anisotropic (*A* bands) and the relatively isotropic bands (*I* bands), which regularly alternate with each other. In general it is held that the polypeptide chains of the *I* bands are permanently folded in contrast to the polypeptide chains of the *A* bands, which are stretched upon relaxation and folded upon contraction of the muscle. Stretched chromosomes and stretched cytoplasmic fibrils are similar to relaxed myofibrils, while cytoplasmic fibrils and chromosomes in normal condition are similar to contracted myofibrils. The *I* bands of muscles are analogous to the chromomeres and chromidia, where the polypeptide chains are permanently folded, while the *A* bands of muscles are analogous to the interchromomeres and the interchromidia, where the

folding of the polypeptide chains is variable. When chromosomes and cytoplasmic fibrils are subjected to strain the polypeptide chains of the interchromomeres and interchromidia only are unfolded. In relaxed muscles the polypeptide chains of the *A* bands are stretched to a maximum and therefore they cannot be extended any more. The polypeptide chains of the *I* bands are folded and therefore only these polypeptide chains are stretched when relaxed muscles are subjected to strain (54). The similarity between *I* bands, chromomeres, and chromidia is also supported by some other facts which are cited below. It has been demonstrated that in microincinerated preparations of resting muscles heavy ash deposits coincide only with *I* bands (41) where adenylnucleic acid is also present (23). This ash consists chiefly of calcium and magnesium. Also within the chromidia and the chromomeres nucleic acid is associated with these bivalent metals.

However, there exists an essential difference between the chromosomes on the one hand and the myofibrils and cytoplasmic fibrils on the other hand. The chromomeres (genes) of the same chromosome differ from each other, while the corresponding sections of cytoplasmic fibrils and myofibrils, respectively, are identical with each other. Probably the same holds true for the interchromomeres on the one hand and the interchromidia and *A* bands on the other hand.

All protein fibrils seem to have a periodic structure and therefore it is probable that not only the cross-striated myofibrils, but also all other fibrils, may be fragmented into their two different component parts. It would be of interest to subject to chemical analysis these two fractions of various fibrils.

C. CORTEX AND PLASMA MEMBRANE

The cortex, which represents the superficial layer of the living cytoplasm, is an essential component of both animal and plant cells. The cortex differs distinctly from the underlying cytoplasm. Moreover, the cortex is entirely different from the extraneous coats covering the cells. In contrast to the cortex, the above-mentioned coats may be removed without injuring the cells (25). The extraneous coats are merely inanimate cellular envelopes. They appear as well-defined membranes or as indistinct layers of various substances of protein or carbohydrate nature. The real plasma surface must be uncovered by means of experimental agents, which fact has been particularly emphasized by Chambers (25).

The ground cytoplasm beneath the cortex is a relatively coarse texture of fibrils oriented in any direction. In many cells this cytoplasmic texture is strongly condensed in the peripheral region and loosened in the central region. Therefore the peripheral part of these cells is a pronounced jelly in contrast to the fluid interior. Chambers refers to this peripheral cytoplasmic region when using the term "cortex." This peripheral cytoplasm is also called ectoplasm, particularly if it is deprived of all inclusions. There is, however, no essential difference between the ectoplasm and the entoplasm. The only difference is the degree of condensation of the cytoplasmic texture. In this article the term cortex is not used in the same sense as Chambers uses it. The real cortex is sharply delimited and it is very different from the jelly-like cytoplasm underlying the cortex. The term cortex is used here to designate a peripheral, very thin, birefringent, jelly-like layer of the cytoplasm. The thickness of this layer is about $1\ \mu$. The cortex maintains the integrity of the whole cytoplasm. The substance of the cortex is much more condensed than the substance of the cytoplasm underlying the cortex.

Birefringence on the surface of various cells has been demonstrated by several authors (Schmitt, Bear, Ponder, Chinn, Schmidt, *et al.*; for literature, see 140). Of particular interest are the investigations of Schmitt, Bear, and Ponder dealing with the induced birefringence of the stromata of hemolyzed erythrocytes of mammals. It was found that this induced birefringence is due, in some cases, to the lipide and in other cases to the protein component of the stromata. Nevertheless, further detailed investigations were necessary in order to elucidate the two questions: (1) Whether the birefringence demonstrated on the cellular surface is always due to the real surface layer of the cytoplasm or whether this birefringence is sometimes simulated and concealed by the birefringence of some extraneous coat; and (2) whether a distinct birefringence may be exhibited by the real surface layer of the living cytoplasm of typical cells, such as sea urchin eggs which are not injured and not subjected to any experimental treatment. Investigations of these questions have been performed by Runnström, Monné, and Broman (129), Runnström and Monné (127), Monroy and Monroy-Oddo (106), and Monné (102-104).

The surface of living uninjured sea urchin eggs not subjected to any experimental treatment exhibits a distinct birefringence which is

positive in radial direction (106,127,129). This birefringence remains unchanged when all extraneous coats of the sea urchin eggs are removed by means of trypsin digestion (129). It is known that proteolytic enzymes do not attack and do not injure living protoplasm. It must be added that cleavage takes place upon insemination of eggs treated with trypsin. From these facts indisputable evidence is provided to prove that the birefringence on the surface of sea urchin eggs is due to the cortex only, and not to some extraneous coat intimately adhering to this cortex. The cortical birefringence disappears when the eggs are subjected to the action of lecithinases (bee venom) and various lipid-dispersing agents (detergents, etc.). Thus, it is certain that this birefringence is due to lipides. The sign of birefringence of the cortex is reversed when the proteins are precipitated subsequent to the dissolution of the lipides. From these facts it is concluded that the rod-shaped lipid molecules are oriented perpendicular to the surface of the cortex and the polypeptide chains of the proteins tangential, in any direction within the plane of the cortex. The cortex consists of protein foils and lipid lamellae regularly alternating with each other.

Not in sea urchin eggs only, but also in several other cells it has been found that the cortex is positively birefringent in radial direction (101,102,104). This also holds true for the living uninjured erythrocytes of the polychaete *Glycera rousi* (99). This birefringence is reversed upon hemolysis. No doubt the cortex has a similar structure in all kinds of cells.

It is possible that the cortex is constructed of fibrils which are similar to the fibrils of the underlying cytoplasm (see page 11). The cortex is much denser than the underlying cytoplasm and therefore it is probable that the cortical fibrils are much thinner than the cytoplasmic fibrils. The structure of both kinds of fibrils may, however, be similar. This statement can be supported by some facts. It has been found that in microincinerated preparations of various cells heavy ash deposits are always present on the site of the cell surface (142). This ash contains chiefly calcium and magnesium. It is known that these ash deposits occur wherever large amounts of nucleic acid are present. Thus, it is possible that some kind of nucleic acid is also present within the cortex. At any rate, adenylnucleic acid was found within the surface layer of the cytoplasm of plant cells (85). Therefore it is possible that the cortical fibrils also consist of nucleic-acid-containing and nucleic-acid-free sections regularly

alternating with each other. However, the rod-shaped lipid molecules must be oriented perpendicular to the polypeptide chains of the cortical fibrils. The polypeptide chains of the cortical fibrils which are oriented in tangential direction constitute the protein foils, and the lipid molecules oriented in radial direction represent the lipid lamellae of the cortex. It is probable that also in the case of the cortex the phosphoric acid parts of the phosphatides and the nucleic acids are kept together by means of the bivalent cations, calcium and magnesium. The enzymes, particularly the enzymes of the energy-yielding activities, must be associated with the nucleic-acid-containing sections, as in the case of the cytoplasmic fibrils (see page 33). The meshes between the cortical fibrils represent the pores which are important for the permeability phenomena.

The structure of the cortex is very similar to the postulated structure of the hypothetical plasma membrane. Nevertheless the cortex was calculated to be 1000 m μ thick in contrast to the plasma membrane which was estimated to be only 10 m μ thick (see 66). The last-mentioned estimation, which is based on various experiments, is, however, not very reliable. It is not certain whether the cortex is identical with the plasma membrane. It may be that the plasma membrane only corresponds to the superficial layer of the cortex. At present it is impossible to decide whether the selective permeability is due to the whole cortex or to its superficial layer only.

D. MITOCHONDRIA, GOLGI BODIES, AND CHLOROPLASTS

Bensley and Hoerr (7,67) were the first to succeed in separating mitochondria from minced tissues. Since then numerous investigations on the chemical composition of the extracted mitochondria have been performed (for literature, see 42). Moreover, numerous cytochemical reactions have been tested. The mitochondria contain proteins, phosphatides, cholesterol, glycerides, respiratory enzymes (7,27,34, and others) amylase (69), and vitamins A and C (for literature, see 11). The amount of cholesterol present within the mitochondria is higher than in any other cellular component (34). In fixed preparations it was found that the mitochondria do not absorb ultraviolet light (2600 Å) to any considerable degree (57,78). This means that no considerable amount of nucleic acid can be present within the mitochondria. This result is more convincing than the result of certain chemical analyses performed on extracted mitochondria, where considerable amounts of ribonucleic acid could be demonstrated (27). Evidently these extracts were still strongly con-

taminated with the chromidial substance which contains large amounts of this acid.

Short rod-shaped and long filamentous mitochondria are positively birefringent in longitudinal direction (51,97,138). This birefringence is due to the protein component of the mitochondria.

Up to the present nobody has succeeded in extracting Golgi bodies from minced tissues. Investigations on this cytoplasmic component have been performed on living and fixed tissues only. For literature concerning this subject the reader is referred to the books of Cowdry (30) and Hirsch (64).

The Golgi apparatus is a universal component of the cytoplasm. It exhibits a certain morphologic, physical, and chemical variability, just as any other cellular component of widespread occurrence. In particular, there exist some important although not essential differences between the Golgi bodies of germ and tissue cells, and between the Golgi bodies of vertebrates and invertebrates. In the author's opinion all these differences are explained if it is assumed that the amount of proteins present within the Golgi bodies is variable and that cholesterol is in some cases present and in others absent; the phosphatides are the essential and most important chemical constituent of this cytoplasmic component.

In living germ cells (spermatocytes, oocytes) of various invertebrates the Golgi bodies may be distinctly seen by means of the ordinary microscope. The phase contrast microscope is a splendid method for detecting Golgi bodies in these cells (15,102). The Golgi bodies appear black on a dark ground. They are distinctly optically differentiated from the surrounding cytoplasm probably because they contain some amount of cholesterol which is a highly refringent substance and which, moreover, has the property of strongly condensing phosphatide films. These Golgi bodies also appear to be relatively rich in proteins. The Golgi bodies in the above-mentioned cells (*e.g.*, male germ cells of pulmonates and chilopodans) are birefringent (91,94). Their birefringence may be greatly increased under the influence of hypertonic salt solutions and vital staining with chrysoidine (94). Moreover, in fixed preparations these Golgi bodies are easily stained with Heidenhain iron alum hematoxylin, particularly at the temperature of 37°C. This staining may be due to the presence of cholesterol within the Golgi bodies. It is known that iron alum, which combines with hematoxylin, is easily reduced by chole-

terol at this temperature. The mitochondria which do not contain any considerable amount of nucleic acid, but large amounts of cholesterol, are deeply colored when this method is applied.

In contrast to germ cells, the Golgi bodies are, with few exceptions, invisible in living tissue cells and they cannot be demonstrated either by means of the phase contrast or the polarization microscope. Moreover, in fixed preparations the Golgi bodies of these cells cannot be stained with iron hematoxylin. They may be detected only by impregnation with osmium and silver; these methods are also successfully applied to demonstrate Golgi bodies in germ cells. The Golgi bodies of tissue cells probably do not contain any cholesterol and that may be the reason they are not optically differentiated from the surrounding cytoplasm. Moreover, they are very poor in proteins. Under the influence of various experimental agents the ground cytoplasm and the Golgi bodies become simultaneously birefringent and therefore they cannot be distinguished from each other. In spite of these differences the Golgi bodies may have the same structure in both germ and tissue cells. When living tissue cells are exposed to high-speed centrifuging, the Golgi bodies are displaced, which may be demonstrated in fixed preparations (5). This is indisputable evidence that the Golgi bodies are real components of the cytoplasm even in the cells, where they are invisible in living condition.

The Golgi bodies have the shape of platelets, lenses, globules, hemispheres, and invaginated gastrula-like bodies. Net-like Golgi apparatus has never been observed in living cells. It is very probable that net-like Golgi apparatus is a fixation artifact due to fusions and alterations of the single Golgi bodies. This is strongly supported by the model experiments of Holtfreter (71). The Golgi bodies always consist of two components: the externum and the internum. The former is membranous while the latter is globular or lens shaped. The interna are always compact and therefore they cannot be regarded as vacuoles.

It is universally accepted that large amounts of phosphatides are present within the Golgi bodies. Osmium tetroxide is strongly reduced only by the externa of the Golgi bodies. Moreover, under the influence of various agents, only the externa are transformed into myelin figures at room temperature (92). Both facts indicate that phosphatides with unsaturated fatty acids in their molecules prevail within the externa of the Golgi bodies, and phosphatides with saturated fatty acids within the interna. It is supposed that cholesterol may or may not be present within the Golgi bodies. Moreover, small

but variable amounts of proteins are present within the Golgi bodies. The latter do not absorb ultraviolet light to any considerable degree and therefore they cannot contain any considerable amount of nucleic acid (63). In microincinerated preparations no distinct ash deposits are found in the region occupied by the Golgi apparatus (142). Thus, it is very poor in minerals, which is also evidence that it does not contain nucleic acid (see page 7).

Neutral red is a vital stain which affects the Golgi bodies. Vacuoles deeply stained with this dye appear, as a rule, in the region of the cytoplasm which is occupied by the Golgi bodies. Moreover, it may be demonstrated that neutral-red vacuoles are formed in association with the Golgi bodies, when the latter are visible in living cells. The externa of the Golgi bodies are only exceptionally stained with this dye. Neutral red is strongly accumulated within the interna of the Golgi bodies, where local cytolysis is produced. The lipides are separated from the proteins and a vacuole stained with neutral red is exuded. Thus, the Golgi apparatus is vacuolized under the influence of neutral red. A similar effect is produced by morphine (102). The mentioned neutral-red vacuoles are pure artifacts, which do not correspond to any real component of the cytoplasm. Also this conclusion is strongly supported by the model experiments of Holtfreter (71).

The lamellar externa of the Golgi bodies of living germ cells of invertebrates are or may easily be made birefringent (91,94). The birefringence is positive in the direction which is perpendicular to the surface of the lamellae. This means that the rod-shaped lipide molecules are also oriented perpendicular to the surface of the lamellar externa. The globular interna of the Golgi bodies of the spermatocytes and spermatides of *Lithobius* are positively birefringent in radial direction (94). Thus, the rod-shaped lipide molecules of the interna must also be oriented in this direction. The Golgi bodies should be regarded as systems of lipide lamellae and protein foils regularly alternating with each other. The above-mentioned foils must be very thin, because the protein content of the Golgi bodies appears to be very low.

The chloroplasts of plant cells are also systems of alternating protein foils and lipide lamellae. They show a similar, although stronger, birefringence than the Golgi bodies (for details and literature, see 45,87).

E. ORGANIZATION OF THE CELL

The relatively firm cortex covers the underlying ground cytoplasm. The latter consists of the cytoplasmic fibrils (see page 4) and the enchylema. The term enchylema has been employed by previous cytologists to denote the liquid which fills the interstices between the fibrillar components of the protoplasm. The ground cytoplasm is not a network of single polypeptide chains; it is a relatively coarse texture of cytoplasmic fibrils which are bundles of numerous polypeptide chains. The cytoplasmic fibrils are not interconnected by means of transverse threads. This would prevent the previously described (see page 11) regular parallel conjugation of the cytoplasmic fibrils. The ground cytoplasm is not a network, but rather a texture of fibrils (98,100,119). The cytoplasmic fibrils adhere weakly to each other by means of lipide molecules perpendicularly oriented to their length. The cytoplasmic fibrils adhere somewhat more strongly to each other by means of their protein constituents. These lateral linkages between the protein molecules are brought about by a process similar to blood clotting (see page 28).

Within the enchylema various inclusions are suspended, such as the small, globular or rod-shaped mitochondria, yolk, free glycogen, and many others. These inclusions move when the enchylema is induced to flow. Thus, the small globular or rod-shaped mitochondria do not generally adhere to the cytoplasmic fibrils. Nevertheless, it is possible that under certain physiologic conditions the small mitochondria are reversibly attached to the cytoplasmic fibrils, so that long thread-like structures (chondrioconts) are produced.

The living cytoplasms and the living nuclei are practically neutral, their *pH* being fairly constant (for literature, see 59 and 144). Small deviations from the point of neutrality (*pH* 6.8 to 7.0 for the cytoplasm, *pH* 7.5 to 7.6 for the nucleus) may be due to the slight injury caused by microinjection of indicator dyes. No doubt only the *pH* of the buffered enchylema is in question here. Moreover, there is strong evidence that the electrical charges of the fibrillar components of the living protoplasm are neutralized by inorganic anions and cations, but chiefly by phosphatides in association with the bivalent calcium and magnesium ions (see p. 50). Temporary electrical charges seem to appear only upon stimulation. Permanent electrical charges can be demonstrated in fixed preparations when the cells are preserved in lipide-dissolving fixatives (99,100). Large amounts of

lipides are present both within the cytoplasm and the nucleus (152).

The architecture of the cytoplasm is variable because the distribution of cytoplasmic fibrils is different in different cells. This structure is, however, very plastic; it may easily be disturbed and later reconstituted. Reversible, experimentally produced alterations of the cytoplasmic architecture of the sea urchin eggs have been observed by Runnström (123,125), and thoroughly investigated by Monné (98,100,103). In large, globular, undifferentiated cells the cytoplasmic fibrils may be irregularly distributed in all directions. In some cases, however, tangential orientation of the cytoplasmic fibrils weakly prevails (94). There exists in these cells a certain tendency to form concentric cytoplasmic layers. Radial orientation of the cytoplasmic fibrils may prevail during mitosis. A spiral arrangement of these fibrils was also observed (101). In elongated cells the cytoplasmic fibrils tend to be oriented in the direction of the largest extension. In greatly flattened cells the cytoplasmic fibrils are oriented in any direction but always parallel to the largest surface.

The texture of cytoplasmic fibrils may be either uniform or condensed in certain regions and loosened in other regions of the cell. The condensed cytoplasmic texture may form a layer of variable thickness, contiguous with the cortex. This layer may be free from all inclusions when the cytoplasmic texture is strongly condensed. A similar layer may be formed on the surface of the nuclear membrane. Several concentric alternating layers of condensed and loosened cytoplasm may be present. The cytoplasmic texture is frequently condensed around the centrosomes. The polar and dorsoventral organization of various eggs is due to unequal condensation of the cytoplasmic texture (28,98,101,121). The ergastoplasm is a local condensation of the cytoplasmic texture. It may have the form of long streaks, spirals, and concentric lamellar bodies known as yolk nuclei.

Gram-positive and Gram-negative bacteria differ greatly from each other in their morphologic and physiologic properties. The protoplasm of the former is differentiated into an interior core and an exterior layer, the latter containing large amounts of ribonucleic acid (see 37). The fibrillar ribonucleic-acid-containing components of the cytoplasm appear to be strongly condensed within the exterior layer of the Gram-positive bacteria as in the case of certain eukaryote cells (see 100, Pl. 1, Fig. 12). Cytoplasm of Gram-negative bacteria does not exhibit this differentiation probably because the ribonucleic-

acid-containing fibrils are uniformly distributed throughout the whole cell.

The cell is an organized system of several kinds of self-perpetuating fibrils probably all consisting of nucleic-acid-free and nucleic-acid-containing sections regularly alternating with each other, the latter being associated with enzymes, particularly the enzymes of the energy-yielding activities (see page 33). In the case of undifferentiated cells the most important fibrillar systems are the chromosomes, the cytoplasmic fibrils, the cortical fibrils, and the caryoplasmic fibrils (the latter occupy the interstices between the chromosomes of the resting nuclei). The specific fibrillar systems of differentiated cells are the myofibrils, neurofibrils, epithelial fibrils, cilia, flagella, etc.

IV. Functioning of Cytoplasm

A. DYNAMIC STATE OF THE STRUCTURE OF PROTOPLASM

Living protoplasm is continuously in a dynamic state. Energy is required to maintain the structure of the protoplasm, to bring about the cyclic structural alterations underlying any specific function, to produce various structures during embryologic development, to reconstitute the structure during regeneration, and to modify, within certain limits, the structure of the protoplasm in adaptation to changing environmental conditions.

Protoplasm is a highly organized colloidal system. Colloids are known to change their properties spontaneously. Syneresis and hysteresis occur. This quality of the protoplasmic colloids is regarded to be the cause of aging (132). The colloidal structure is not permanent. It breaks down spontaneously. The structure of the protoplasm would also soon be impaired and would break down spontaneously with ensuing cytolysis if there were not a mechanism present tending to prevent it. This mechanism is the metabolism by which the living substance is continuously broken down and reconstituted. Thereby the protoplasmic colloids are continuously rejuvenated. For this reason the protoplasm is potentially immortal. Aging of the protoplasm of single cells is prevented and aging of multicellular organisms is delayed. This metabolism is not stimulated in inactive organs, which therefore atrophy. In these organs the living substance is not renewed to the same extent as it is impaired spontaneously as a consequence of the metastability of its colloids. This occurs for some reason in multicellular organisms and therefore

aging is caused. The continuous breakdown and reconstitution of the chemical constituents of the protoplasm must be regarded as an established phenomenon well supported by the experiments on cellular metabolism performed by the use of labeled (radioactive) elements (for literature, see 141). Runnström (124,125) and Monné (103) demonstrated that the structure of the cytoplasm of sea urchin eggs is altered when the respiration of the eggs is depressed by various agents, particularly potassium cyanide and sodium azide. Moreover, it has been emphasized by the former investigator that fermentation (glycolysis) is not sufficient for the maintenance of the normal structure of the cytoplasm of the sea urchin egg. Nevertheless, this does not mean that fermentation is without importance for the maintenance of this structure. Fermentation must suffice for the maintenance of the normal structure of protoplasm in the case of organisms which normally live under anaerobic conditions. It is known that nerve cells of warm-blooded animals are very sensitive to lack of oxygen. Evidently considerable work must be performed in order to maintain the normal structure of these cells. Not the inhibition of the respiration as such is the cause of cell death, but the irreversible structural alterations of the protoplasm which invariably occur when the energy-yielding cell activities are inhibited. These rapid structural alterations cannot be due to the metastability of the protoplasmic colloids only, but to the appearance of certain detrimental substances or to the disappearance of other substances necessary for the maintenance of the normal structure of the protoplasm. Evidently energy is required to produce or to maintain the former substances and to remove or inactivate, in some way, the latter. Probably the cytoplasmic fibrils perform continual active movements which cease when the energy-yielding activities are inhibited. The cessation of these movements and the permanent stretching of the polypeptide chains of the cytoplasmic fibrils may also contribute to the appearance of these structural alterations.

It is universally accepted that the protoplasm is a soft thixotropic gel (24,144) which continuously changes its state under physiologic and experimental conditions. Nevertheless the thixotropy of the protoplasm is not a passive physical thixotropy, but an active biological thixotropy controlled by several antagonistic substances, particularly enzymes. Some of these substances present within the cell tend to liquefy the protoplasm by breaking down fibrils, while other

substances in the cell tend to solidify the protoplasm by building up fibrils and by producing lateral linkages between these fibrils. Therefore the structure of the protoplasm cannot be seriously disturbed by microneedles. The fibrils are reconstituted almost at the same moment as they are cut through. The solidifying agents may be similar to enzymes and other substances concerned with blood clotting. The presence of this clotting system within the protoplasm has been particularly postulated by Heilbrunn (59). Meiotic chromosome pairing, the above-described conjugation of cytoplasmic fibrils, the reunion of chromosome fragments, and the formation of membranes and of cytoplasmic nodes may be processes similar to blood clotting. The viscosity of the cytoplasm is increased when the number of lateral points of attachment [*Haftpunkte* of Frey-Wyssling (45)] between the protein components of fibrils is increased (p. 24).

Runnström (124,125) and Monné (103) demonstrated that the structure of the cytoplasm of sea urchin eggs coarsens when respiration is inhibited. Obviously respiration is necessary in order to control the clotting system of the protoplasm. Death occurs when this clotting is strong. The spotted or striated appearance of neurons of warm-blooded animals is due to condensation of the cytoplasmic fibrils which consist of the ribonucleic-acid-containing chromidia (Nissl substance) and the ribonucleic-acid-free interchromidia alternating regularly with each other. It is evident that the clotting and coarsening of the structure of the cytoplasm of the neurons occur rapidly when respiration ceases at the moment of death of warm-blooded animals. It is of interest to note that this change does not occur in excessively stimulated neurons. In these cells the structure of the cytoplasm does not coarsen, the fibrils do not agglutinate with each other, and consequently, the chromidia remain diffusely distributed. A similar phenomenon has been observed by Runnström and Monné (128) in sea urchin eggs. Clotting and coarsening of the structure of the cytoplasm are brought about much more easily in mature unfertilized than in fertilized eggs. The latter are stimulated to development and therefore they are comparable with the excessively stimulated neurons. In cells whose activity is depressed, the structure of the cytoplasm is coarse or it may easily be made coarse by means of experimental agents.

Energy is required to produce the cyclic changes of the microscopic and submicroscopic structure underlying any specific function. The

protoplasm is an organized system of several kinds of living fibrils, such as chromosomes, caryoplasmic fibrils, cytoplasmic fibrils, cortical fibrils, myofibrils, neurofibrils, epithelial fibrils, cilia, flagella, etc. It is universally accepted that muscle contraction is due to the folding of the polypeptide chains of the myofibrils.* Nevertheless, it is highly probable that several other specific kinds of folding of polypeptide chains are the essential phenomena underlying many other functions of protoplasm, *e.g.*, ciliary movements, active transport of lipide-insoluble substances across the plasma membrane (104), nerve conduction (103), movements of chromosomes, mitosis, etc. Thus, folding and unfolding of polypeptide chains seem to be the essential features of the cyclic structural alterations underlying any specific function of the protoplasm. No doubt, motility and contractility, which are due to active folding of polypeptide chains, are the characteristic life phenomena of all living fibrils. Energy is needed to produce the folding of polypeptide chains, or to increase or decrease the degree of folding of the polypeptide chains of various kinds of fibrils. The folding of the polypeptide chains of the fibrils may proceed in several different ways. The folding and unfolding of the polypeptide chains of the whole fibril may occur simultaneously or it may proceed in waves. In the former case the whole fibril is shortened and stretched. In the latter case the fibril may remain apparently unchanged. The waves may be either long or short. They may move slowly or rapidly. The folding of polypeptide chains may be either strong or weak. Finally the folding capacity may be different in different parts of fibrils. Thus, it seems that a great variety of protoplasmic functions have a common foundation. The specific functions are supposed to be merely modifications of one basic function common to all living fibrils, that is, active folding of polypeptide chains. In the following sections an attempt will be made to prove this hypothesis.

It is a well-known fact that the rate of respiration is increased upon injury, *e.g.*, the rate of respiration of sea urchin eggs is increased under the influence of hypertonic sea water (124). Thus, energy is made available to reconstitute the normal structure of the cytoplasm

* Szent-Györgyi (6th International Congress of Experimental Cytology, Stockholm, 1947) advanced the view that the contraction of myofibrils is due to spiralization of polypeptide chains. This hypothesis may hold true for all kinds of protoplasmic fibrils.

or to modify this structure within certain limits in adaptation to changed environmental conditions.

B. CATABOLIC ACTIVITY

The catabolic activity of the cell is due to enzymes. It is indisputable that the protoplasm is not a random mixture of a great variety of enzyme molecules. Metabolism is a regular sequence of chemical reactions which occur at the right time and the right place, and which are appropriately modified in adaptation to changing environmental conditions. Therefore, the protoplasm must be regarded as an organized system of enzymes whose activity is strictly controlled. The enzymes are activated and inactivated according to the needs of the living cell. The enzymes must be concatenated with each other and arranged in certain patterns. The harmonious collaboration of enzymes is maintained by the ordered structure of protoplasm. This collaboration is disturbed when the structure of the protoplasm is altered. The task of the cytologist is to find out how the enzymes are distributed within the protoplasm.

The distribution of enzymes may be investigated by means of two methods: cytochemical reactions on tissue sections and testing of the enzymic activity of various isolated cellular components. Unfortunately most of the cytochemical reactions performed on frozen or fixed tissue sections are either not sensitive enough or are highly unreliable (32). Cytochemical methods have been elaborated for the demonstration of the following enzymes: phenolase, peroxidase, dopa oxidase, urease, acid phosphatase, alkaline phosphatase, and lipase. Gomori (49) found that some of these methods are valuable. The method of separating various morphologic components (7) of the cell and testing their enzymic activity is much more important and has already yielded very interesting results. Nevertheless, reliable results can only be obtained when the preparations are not contaminated with any other substance. A number of contradictory results indicates that this was not always the case.

It is a well-established fact that certain enzymes, particularly a number of the most important enzymes of respiration and fermentation, are firmly bound to cellular structures, in contrast to other enzymes which are either free or only weakly associated with these structures (38). It is known that the energy-yielding activities are not inhibited when living cells are minced, and when autolysis, de-

naturation, and similar alterations of the cellular fragments are prevented simultaneously. Since the time of Warburg's (154) investigations it has been known that respiration is exhibited by very small granules extracted from various cells. These granules correspond to the mitochondria and chromidia which have been previously discovered on fixed preparations. Thus, it is certain that respiratory enzymes are contained within these cytoplasmic components.

Within the chromidia (microsomes) various enzymes of the energy-yielding activities have been found, such as cytochromes a and b, cytochrome oxidase, succinic dehydrogenase, peroxidase, and catalase (Stern, Ballentine, Jeener, Brachet, Chantrenne, Lazarov, *et al.*; for literature, see 13 and 42). The two first-named enzymes are firmly bound to the granules. The remaining enzymes are not so strongly bound and therefore they are partially isolated when the granules are extracted from the cells. Recently some doubts have been expressed by Claude (27) as to whether the presence of the respiratory enzymes within the chromidia (microsomes) has been proved. It is possible that the method used by this author prevented him from detecting these enzymes. Moreover, a great variety of hydrolyzing enzymes has been found to be present within the chromidia such as acid and alkaline phosphatases, ribonuclease, amylase, dipeptidase, trypsin, cathepsin, arginase, and adenylic acid deaminase (Brachet and Jeener, Chantrenne, Steinbach and Moog, *et al.*; for literature, see 13,42). The hydrolyzing enzymes are weakly attached to the chromidia.

It has been demonstrated by Monné (98,100,104) that the chromidia, which contain ribonucleic acid, are concatenated with each other by means of the interchromidia, which are free from this acid. Thus the chromidia are integral components of the cytoplasmic fibrils. The important enzyme adenosinetriphosphatase has been extracted from a great variety of cells (for literature, see 117). Thus, it does not occur only in cross-striated muscle: it is a general chemical constituent of protoplasm. This enzyme is identical with myosin, the birefringent contractile protein of muscles (29a,39,118).

Potter advanced the view that adenosinetriphosphatase might be identical with the substances that exhibit streaming birefringence when separated from various cells. Monné (104) arrived at the conclusion that adenosinetriphosphatase must be present within the cytoplasmic fibrils because they are contractile and because they are

the only constituents of the ground cytoplasm which exhibit birefringence in living cells. Steinbach and Moog (150) found that this enzyme is present within small cytoplasmic granules extracted from minced cells. Nevertheless, they (Steinbach and Moog) did not investigate whether streaming birefringence is exhibited by these cellular extracts. Undoubtedly the cytoplasmic fibrils are fragmented into chromidia and interchromidia when the cells are minced. It is possible that the preparations of the chromidia investigated by Steinbach and Moog (150) were contaminated with interchromidial substance. Nevertheless, it is probable that adenosinetriphosphatase is present in both the interchromidia and chromidia. In this case it must be assumed that the birefringence of the adenosinetriphosphatase is compensated by another substance only within the chromidia. This conclusion is supported by the recently discovered fact that the isotropy of the *I*-bands of cross-striated muscles is entirely due to compensation (86a).

Thus chromidia are organized systems of a great variety of molecules. They contain proteins rich in sulfhydryl groups, ribonucleic acid, calcium, magnesium, phosphatides and other lipides, and respiratory and hydrolyzing enzymes. All these constituents of chromidia must be linked together in an ordered way, so that the energy released during the oxidation of a certain substance may be used for the synthesis of another substance. Evidence is accumulating that nucleic acid is necessary for protein synthesis. The presence of ribonucleic acid proves that living substance is synthesized within the chromidia. There is no doubt that within the chromidia the catabolic activities are intimately linked with the anabolic activities of the cell. The ordered concatenation of chemical reactions must find its explanation in the structure of chromidia. There is no evidence that the chromidia of a certain cell differ from each other (see page 11). It is probable that all chromidia of a certain cell have the same composition. Thus, practically all enzymes, present within the ground cytoplasm of a certain cell, must be represented within any chromidium. Like genes and viruses, which are also nucleoproteins, chromidia are the ultimate units of life, long since postulated by many investigators (Herrmann, Pflüger, Verworn, Altmann, Heidenhain, *et al.*; literature, see 58 and 61). Chromidia have all the essential properties of living matter. They grow, proliferate, have a certain metabolism and most probably irritability (see page

44). The most primitive viruses exhibit only one of these properties. They grow and proliferate, but they do not exhibit any energy-yielding activity (see 148) or irritability. The energy necessary for the life of viruses is provided by the host cells.

Mutations are a beautiful example of the correlation between structure and function. They occur whenever a structural rearrangement of the chromosomes takes place. Chromidia, which are connected with each other, may influence each other's activity. In contrast to the chromosomes the cytoplasmic fibrils have the same properties along their whole length. Nevertheless, it is possible that structural changes of the cytoplasm are accompanied by changes in the function of the chromidia. These functional changes of the chromidia may be due to fragmentation and elongation of the cytoplasmic fibrils, to loosening and condensation of the cytoplasmic texture, and to the formation of various structural patterns.

The cell is an organized system of several kinds of fibrils, all of which probably consist of two regularly alternating sections, either containing or being devoid of some kind of nucleic acid. In the case of the cytoplasmic fibrils it is certain that respiratory and hydrolyzing enzymes are present within the nucleic-acid-containing sections. Thus, it is also probable that all other protoplasmic fibrils have a similar constitution, their nucleic-acid-containing sections being associated with respiratory and hydrolyzing enzymes and their nucleic-acid-free sections only with adenosinetriphosphatase. It would be extremely interesting to know whether respiratory and hydrolyzing enzymes are accumulated within the chromomeres (genes), within the basophilic sections of the karyoplasmic fibrils, and within comparable components of the fibrils which constitute the cortex. The size of the microsomes separated from various cells varies between 50 and 300 $m\mu$ and therefore it is probable that these microsomes are the product of fragmentation of several kinds of protoplasmic fibrils. This problem should be considered by cytochemists. In this connection it is important to note that up to the present the following enzymes have been found to occur within the nuclei: cytochrome oxidase, lactic acid dehydrogenase, D-amino acid oxidase, choline oxidase, arginase, dipeptidase, esterase, uricase, ribonuclease, acid and alkaline phosphatases (35,77; for literature, see 13).

Mitochondria were found to contain some respiratory enzymes (27,79) and amylase (69). Moreover, some microchemical reactions

indicate that glutathione and vitamin C are also present in mitochondria (for literature, see 11). Mitochondria, which are immersed within the enchylema, circulate freely among the cytoplasmic fibrils whose chromidia are known to contain proteins rich in sulfhydryl groups (13). Glutathione and vitamin C present within the mitochondria may regenerate these sulfhydryl groups and thereby activate the enzymes of the chromidia. It is very probable that enzymes of respiration and other energy-yielding activities are also present within Golgi bodies and chloroplasts, which are, undoubtedly, living, metabolizing components of the cytoplasm.

Free and bound glycogen was found to occur in various cells (82,110,157). The former is deposited in the interstices between the cytoplasmic fibrils and therefore it may easily be extracted from the cells. The latter cannot be extracted so easily, undoubtedly because it is firmly bound to the cytoplasmic fibrils. Bound glycogen is probably present only within the chromidia where it is broken down by enzymes and probably also resynthesized again.

Thus, it is concluded that respiratory and hydrolyzing enzymes occur within the whole protoplasm. The molecules of these enzymes are, however, not mixed at random, but bound to certain morphologic constituents of the protoplasm and arranged in a certain order. Nucleic-acid-containing bodies, fibrils, and cells are the three structural and physiologic units of living beings. Self-perpetuating granules are arranged into self-perpetuating fibrils, and self-perpetuating fibrils are arranged in self-perpetuating cells. The latter may be arranged in multicellular organisms. The chromosomes are self-perpetuating fibrils. Long ago it was supposed that other living fibrils are also able to reproduce themselves by longitudinal division (58). The higher the organization the more complicated the life phenomena. Viruses, which are pure nucleoproteins, exhibit only anabolic activity in contrast to chromidia, which are also endowed with catabolic activity and irritability. Living fibrils exhibit, in addition, contractility and conductivity. The life phenomena of cells and multicellular organisms are even more differentiated.

Living substance is continuously broken down and reconstituted again. Protoplasm is in a state of rapid flux. Not the living substance as such, but its structure, *i.e.*, the constellation of its atoms, is perpetuated. The structure of protoplasm is in a steady state of dynamic equilibrium. This is a well-established fact, evidenced

chiefly by the numerous investigations on cellular metabolism performed by the use of radioactive elements (for literature, see 141). No doubt, energy is required to reconstitute the living substance which is continuously broken down. Even nucleic acid, which is associated with genes (chromomeres) and gene-like components of the cytoplasm (chromidia), is continuously broken down and reconstituted. The same holds true for the cytoplasmic and nuclear phosphatides (62).

Enzymes and other substances constituting the chromidia and similar self-perpetuating particles must be arranged in a certain order. It must be assumed that within the chromidia the nucleoproteins, associated with the enzymes of respiration and other energy-yielding activities, are separated by means of lipides from the hydrolyzing enzymes. This conclusion is supported by the following facts: In general it is held that nucleic acid is concerned with the growth of the protoplasm and particularly with the synthesis of its proteins (13,19). Energy is required for the synthesis of the living substance and therefore it must be assumed that the nucleoproteins are associated with the enzymes of respiration (and other energy-yielding activities) and concatenated with each other in a certain order. The living substance, which is continuously synthesized within the nucleic-acid-containing corpuscles, is also continuously broken down under the influence of hydrolyzing enzymes. Thus, there must exist a continuous antagonism between the anabolic activity of the nucleoproteins and the catabolic activity of the above-mentioned enzymes.

A continuous struggle is displayed by the nucleoproteins and enzymes. In living cells lipides prevent the nucleoproteins from being completely broken down by the enzymes. This is supported by the following facts: The essential feature of cytolysis is the separation of the proteins from the lipides (J. Loeb, see 83). It has been demonstrated by the use of the polarizing microscope that the lipides are present within the whole cytoplasm. The birefringence of cytoplasm, which is due to lipides, is completely abolished upon cytolysis (102,106,129). Evidently the lipides are dispersed. Simultaneously the hydrolyzing enzymes are activated and autolysis occurs. It is known that even nucleoproteins are broken down in autolyzing cells (17). Thus, it is evident that the nucleoproteins are protected by means of lipides against the hydrolyzing enzymes. Autolysis occurs

when this lipid layer is irreversibly broken down. In living cells this protecting lipid layer is present. Living substance is continuously broken down and reconstituted again probably because this protecting lipid layer is also continuously broken down and reconstituted very rapidly in a certain rhythm.

In cells which have ceased to grow the synthetic power of the nucleoproteins and the disintegrating power of the hydrolases are balanced. In rapidly growing cells the former prevails. Inactive organs atrophy because enzymic breakdown and reconstitution of the protoplasm are not stimulated. The protoplasm disintegrates spontaneously owing to the metastability of its colloids.

From certain experiments the conclusion may be deduced that several energy-yielding enzyme systems are present within the same cells. One of these enzyme systems may produce the energy necessary for the continual reconstitution of the living substance (respiration of rest) while the others produce the energy required to bring about the reversible changes of the microscopic and submicroscopic structure underlying mitosis and various specific functions (excess respiration) such as muscle contraction, ciliary movement, nerve conduction, active transfer of lipid-insoluble substances, etc.

Thus, it has been demonstrated by Runnström (124,125) that under the influence of ethylurethan the rate of respiration is decreased in fertilized and increased in mature unfertilized sea urchin eggs. It may be that ethylurethan, in the concentration employed, stimulated the enzyme system, which is concerned with the continual breakdown and reconstitution of the protoplasm, and inhibited the other enzyme system, which is concerned with mitosis. Recently it was found that the rate of respiration of the fertilized sea urchin eggs can also be suppressed by several other chemicals which do not change the rate of respiration of the mature unfertilized eggs (44). Similar differences have been observed between the resting and excess respiration of the muscle (149). It is probable that several energy-yielding enzyme systems concerned with different cytoplasmic functions are present within the same chromidial granules.

Folding and unfolding of the polypeptide chains of the fibrillar components of the protoplasm seem to be the essential phenomena underlying any specific function of the cell. Passive folding and unfolding is a characteristic trait of any inanimate protein fibril. Living fibrils, however, are associated with the enzymes of respiration (and

other energy-yielding activities) and therefore energy is available for active folding and unfolding of the polypeptide chains. One of the above-mentioned respiratory enzyme systems might be concerned with the continual reconstitution of the substance of these fibrils and the other with the causation of the special kind of folding of the polypeptide chains characteristic of these fibrils.

C. CONTRACTILITY AND MOTILITY

It has long been recognized that contractility is a general property of protoplasm. For literature concerning this subject the books and papers of Schmidt (138,140), Heilbrunn (59), Fenn (43), and Holtfreter (72) should be consulted.

As already pointed out above the cytoplasm is a texture of fibrils consisting of the ribonucleic-acid-containing chromidia and the ribonucleic-acid-free interchromidia regularly alternating with each other. The enzymes of the energy-yielding activities are associated with these fibrils. The polypeptide chains of chromidia are permanently folded, in contrast to the polypeptide chains of interchromidia, which may be easily unfolded and folded again. It has been demonstrated by Monné (103) that cytoplasmic fibrils are stretched under the influence of dehydrating agents. Evidently the polypeptide chains of interchromidia are stretched in the dehydrated, and folded in the hydrated, condition. In living cells the polypeptide chains of cytoplasmic fibrils are folded, the degree of folding being variable under different physiologic conditions.

The structure of protoplasm is in a dynamic condition and therefore it is probable that slow, active movements are continuously performed by the cytoplasmic fibrils. No doubt the degree of folding of the polypeptide chains of cytoplasmic fibrils is actively increased or decreased. Energy necessary to produce these active movements is provided under the influence of enzymes associated with the cytoplasmic fibrils. Folding and unfolding of the polypeptide chains is brought about by various hydrating and dehydrating substances which continuously appear and disappear during cellular metabolism. The same substances may also cause a distension and condensation of the texture of cytoplasmic fibrils.

The structure of cytoplasm is subject to continual changes under the influence of the cellular metabolism. The polypeptide chains

which are stretched in one region may be simultaneously folded in another region of the same fibril. Short folding waves of the polypeptide chains may run along the fibrils. Moreover, in certain regions of the cell, folding, and in other regions, stretching, of the polypeptide chains may prevail. This condition may be subject to rapid changes. The degree of folding of the polypeptide chains of the single cytoplasmic fibrils of the same cell cannot be constant. Nevertheless, the average degree of folding of the polypeptide chains may be constant and it may be actively decreased or increased under different physiologic conditions. Energy is required to produce substances by which hydration and dehydration, folding and unfolding of the polypeptide chains are brought about.

Runnström (122,123) found that the hydration of the cytoplasm of the sea urchin eggs is increased upon fertilization. No doubt the average degree of folding of the polypeptide chains of the cytoplasmic fibrils is also increased. The energy necessary to produce this change is provided by the increased rate of respiration occurring upon fertilization. Nevertheless, energy may also be necessary to decrease the degree of folding of polypeptide chains. This may occur during the formation of the astrospheres. Some of the cytoplasmic fibrils are transformed into the rays of the astrospheres, probably under the influence of a substance which diffuses from the centrosomes. The cytoplasmic fibrils of sea urchin eggs are negatively birefringent (98,100) and the rays of the astrospheres are positively birefringent (122,123,138,140) in longitudinal direction. Evidently, during the transformation into the rays of the astrospheres, the cytoplasmic fibrils are partially deprived of their lipides, dehydrated, and stretched. Thereby, their sign of birefringence is reversed. Energy is needed to produce substances by which hydration and dehydration, and folding and unfolding of polypeptide chains are brought about. The cytoplasmic fibrils need energy in order to bind water and in order to expel this water. It has been stressed, particularly by Rapkine, Mirsky, and Brachet (for literature, see 13), that during mitosis the polypeptide chains are stretched when the cytoplasmic proteins are reversibly denatured.

Chromosomes are able to perform active movements. This is evidenced by meiotic phenomena and by the variability of the position of the chromosomes within the equatorial plates of subsequent cellular divisions. The mechanism of these movements must be the

same as in the case of cytoplasmic fibrils. Similar movements may also be performed by the chondriocents. Moreover, it is probable that the polypeptide chains of the fibrils, constituting the cortex, are also actively folded and stretched (104).

Contraction and distension of the cell is due to two different factors: condensation and distension of the texture of cytoplasmic fibrils, and folding and unfolding of the polypeptide chains of the single fibrils. The latter phenomenon was particularly considered by Schmidt (140) and Fenn (43). It has been demonstrated by Monné (100,103) that, under the influence of strongly hypertonic sea water, the ground cytoplasm of sea urchin eggs contracts and shrinks away from the cortex. The texture of cytoplasmic fibrils is markedly condensed. Simultaneously all inclusions are forced out from the interstices between the fibrils. The single cytoplasmic fibrils are, however, not contracted but, on the contrary, are stretched under the influence of hypertonic sea water. The distances between the chromidia of the single cytoplasmic fibrils are increased because the polypeptide chains of only the interchromidia are stretched. Thus, in this case the contraction of the cytoplasm is entirely due to the condensation of the texture of fibrils. If the eggs were exposed to the action of weakly hypertonic sea water, the contraction due to the condensation of the cytoplasmic texture would be balanced or overbalanced by the simultaneous distension which is due to the elongation of the single cytoplasmic fibrils.

Thus, it is supposed that the mechanism of cytoplasmic contractility is based on an antagonistic principle. The distension of the cytoplasmic texture under the influence of the decreased osmotic pressure of the medium may be balanced, to a certain degree, by the simultaneous contraction and folding of the polypeptide chains of the cytoplasmic fibrils. The condensation of the cytoplasmic texture under the influence of the increased osmotic pressure of the medium may be counteracted to a certain degree by the simultaneous stretching and unfolding of the polypeptide chains of the cytoplasmic fibrils. This may be the mechanism by which the volume of the cell is kept constant within certain limits, when the osmotic pressure of the medium is subjected to certain variations. The penetration of water is limited by the contraction of the cell and the loss of water is limited, to a certain degree, by the distension of the cell. Later, energy may be spent to take up or to force out water against the concentration

gradient and to change the hydration of the cytoplasm in adaptation to the altered osmotic conditions of the medium.

Hydration and dehydration, and condensation and loosening of the cytoplasmic texture are the chief causes of the known cytoplasmic currents. This is supported by the investigations of Conklin (28), Runnström (122,123), Lindahl and Orström (81), Seifriz (144), Monné (98,100), *et al.* The cytoplasmic texture is condensed upon dehydration and loosened upon increased hydration. When this occurs in many different places irregularly scattered within the whole cytoplasm, many weak streamings oriented in all directions are initiated. A rapid cytoplasmic current going in one direction is produced, when the cytoplasmic texture is strongly condensed in one region of the cell and loosened in another. The water, which is released upon dehydration, is mixed with the enchylema, filling the interstices between the cytoplasmic fibrils. The texture of the fibrils is condensed and the enchylema, together with the granules suspended within it, is forced out. The condensation of the cytoplasmic texture seems to be accompanied by the stretching of the polypeptide chains of the fibrils. Currents may be experimentally produced when sea urchin eggs are subjected to hypertonic sea water (100,103). Similar currents may be observed during mitosis (146). The texture of cytoplasmic fibrils is condensed on the poles of the spindle and loosened in the equatorial region. The enchylema, together with the mitochondria, are forced out in equatorial direction. Therefore the mitochondria are accumulated in this region (6). The most beautiful example of this equatorial accumulation has been detected by Poluszyński (116). The rhythmic reversal of the direction of the cytoplasmic currents in slime molds must be due to the rhythmic condensation and loosening of the cytoplasmic texture at the two opposite ends of the plasmodia (144).

It is known that cytoplasmic currents cease when the energy-yielding activities are suppressed. Evidently energy is required to produce condensation and loosening of the cytoplasmic texture. Cytoplasmic currents facilitate cellular metabolism. The products of enzymic activity are rapidly removed and substrates are rapidly supplied. Many cells which exhibit lively cytoplasmic currents do not move and do not change their shape. In other cells, however, these cytoplasmic streamings may produce amoeboid form changes and movements. At least two different mechanisms are operative in the

formation of pseudopodia. The long, thread-like pseudopodia of *Radiolaria*, *Heliozoa*, and *Foraminifera* are positively birefringent in longitudinal direction (140). They are very similar to the rays of the astrosphere. No doubt a similar mechanism is involved in the formation of these thread-like pseudopodia and in the formation of the rays of the astrospheres (see page 38). The stretching of polypeptide chains is considered by Holtfreter (72) to be an important factor in amoeboid movements.

The formation of large lobe-like pseudopodia is due to a totally different mechanism. The reversible change from sol to gel in different regions of the cytoplasm has been regarded to be the cause of the formation of this kind of pseudopodium (see 43). Condensation of the cytoplasmic texture means gel formation; loosening of this texture, sol formation. The enchylema, which is squeezed out from the interstices between the cytoplasmic fibrils in certain regions of the cell, accumulates in other regions, where it distends the texture of the fibrils. The latter regions exhibit the properties of a sol. Granules penetrate the regions of the cytoplasm where the meshes between the fibrils are strongly distended. Amoeboid movements are produced by cytoplasmic currents when the latter reach the cortex and liquefy it reversibly in several places. This conclusion is supported by the investigations of Monné and Wicklund (105), who found that the shape of sea urchin eggs is changed in the most fantastic way under the influence of merthiolate. These amoeboid form changes are accompanied by condensation of the cytoplasmic texture in many different places, whereby the enchylema is forced out and streamings are produced. The cytoplasmic texture is lacerated in the regions where the enchylema is strongly accumulated. Thus, the great instability of the architecture of the cytoplasm is the cause of this kind of amoeboid movement.

In the central regions of finger-shaped pseudopodia the cytoplasmic fibrils are oriented in longitudinal direction; and in the peripheral region of these pseudopodia these fibrils are generally oriented in transverse direction. The cytoplasmic fibrils in the central region are stretched and dehydrated, whereby water is exuded within the tips of the pseudopodia. This water is taken up by the fibrils of the peripheral region of the pseudopodia. These fibrils which are usually oriented in transverse direction are alternately hydrated and dehydrated. Their polypeptide chains are induced to be alternately

folded and unfolded. Therefore annular constrictions move in the direction from the tips to the bases of the pseudopodia. These constriction waves have been investigated in detail by Holtfreter (72). Cell division is due to a similar mechanism. In this case two contraction waves travel from the two opposite poles toward the equator of the dividing cell.

Energy is required to produce hydrating and dehydrating substances which regulate the degree of folding of the polypeptide chains of the cytoplasmic fibrils and the degree of condensation of the cytoplasmic texture. Cytoplasmic streamings and amoeboid movements are produced when, upon stimulation, the equilibrium between these two kinds of substances is disturbed.

The general contractility of the cytoplasm is similar to the contractility of the muscles. Nevertheless, important differences also exist. The cytoplasmic fibrils are similar to cross-striated myofibrils. The polypeptide chains of the anisotropic bands of the myofibrils are stretched in the resting muscle and folded in the contracted muscle. Energy is required to produce this folding. The polypeptide chains of the cytoplasmic fibrils are folded in normal condition. The degree of folding of the polypeptide chains of the cytoplasmic fibrils may be either decreased or increased under different physiological conditions. Energy is required to produce both changes. Moreover, the general contractility of the cytoplasm is due not only to the folding and unfolding of the polypeptide chains but also to the condensation and distension of the texture of cytoplasmic fibrils. The movements of cilia, flagella, and sperm tails are also due to some specific kind of folding and unfolding of polypeptide chains. These movements represent a special kind of contractility.

D. IRRITABILITY AND CONDUCTIVITY

The living cell is able to control its anabolic and catabolic activities. Both activities may be either increased or decreased or modified in adaptation to changing internal and external conditions. The chemical reactions, underlying these activities may be induced, inhibited, accelerated, delayed, or modified. Moreover, various combinations of chemical reactions may be brought about. Under the influence of this metabolism regular changes of the microscopic and submicroscopic structure of the protoplasm are produced. Thus cyclic struc-

tural alterations underlying any specific function are produced; moreover, various structures are formed during embryologic development; while other structures are reconstituted during regeneration and modified in adaptation to changing environmental conditions. There is strong evidence that the catabolic and anabolic activities are controlled by means of lipides. Substances related to lipides may produce a similar effect. A chemical reaction may take place only when lipides and similar substances, which separate the nucleoproteins and the enzymes from the substrates of their activity, are temporarily removed. This lipid barrier may be broken down and reconstituted again according to the needs of the living organism. The facts which support this theory are discussed below.

Hofmeister (68) advanced the view that a great variety of chemical reactions may proceed side by side in the same cell without disturbing each other because the protoplasm is subdivided by means of thin films into numerous smaller regions. Bernstein and Höber (65) concluded that upon stimulation temporary holes are formed within the lipid membranes covering the cells. Lillie (80) claimed that all components of the protoplasm, also fibrils, are covered by films, chiefly of lipid material, which may be temporarily broken down and reconstituted again during the activity of the cell. He assumed also that nerve conduction is due to a similar reversible, rapidly transmitted, structural alteration of the surface film of nerve fibers and constructed ingenious models simulating this phenomenon. Moreover, it has been supposed that bioelectric rhythms are associated with chemical rhythms, both being the expression of changes occurring within protoplasmic membranes. Muralt (109) advanced the hypothesis that the choline arms of phosphatide molecules may perform oscillating movements during nerve conduction. Lillie (80), Biedermann (9), and Runnström (125) claimed that lipides may prevent enzymes from breaking down their substrates. Alsterberg (1) assumed that lipides might be broken down during nerve conduction.

Lipides are present within the whole protoplasm, cytoplasm, and nucleus. The rod-shaped lipid molecules are oriented perpendicular to the fibrillar constituents of the protoplasm. Lipides have been found to be present within the chromidia where most of the enzymes are accumulated. It is known that living uninjured cells are not attacked by hydrolyzing enzymes, in contrast to cytolyzed cells which are easily broken down. Autolysis takes place in the latter case. It

has been demonstrated by Knaff-Lenz and Loeb (see 83) that the separation of the lipides from the proteins is the most essential feature of cytolysis. Even nucleoproteins are broken down in autolyzing cells (17). Thus, it is evident that lipides prevent the proteins, particularly the nucleoproteins, from being broken down by the hydrolyzing enzymes. No doubt, in living cells the nucleoproteins are separated from the hydrolyzing enzymes by means of a lipide layer, which is irreversibly removed upon cytolysis.

It is known that the rate of respiration of sea urchin eggs is frequently increased upon incipient cytolysis (36). Later, however, this respiration ~~is~~ decreased because the respiratory enzymes are destroyed. Runnström (122,123) and Öhman (111) demonstrated that the condition of the lipides is changed when sea urchin eggs are fertilized. Simultaneously ~~the~~ the rate of respiration of these eggs is increased. Thus, it is evident that the enzymes of the energy-yielding activities are also controlled by lipides.* Recently, Barron (3) arrived at the conclusion that: "The rate of reaction of isolated oxidation enzyme systems is extremely high when compared with the rate of respiration in living cells. It must therefore be controlled in the living cells." These enzyme systems correspond to the chromidia separated from minced cells. No doubt the rate of respiration of the isolated chromidia is greatly increased because lipides are, at least partially, lost when these particles are extracted from the cells.

Thus, the above-mentioned facts prove that the activity of all enzymes, including the enzymes of hydrolysis, respiration, and fermentation, is controlled by means of lipides. Evidently the enzymes collected within the chromidia are separated from their substrates by means of lipide molecules. It is supposed that upon stimulation the condition of the lipides is changed. Probably the lipides are temporarily pushed away from the surfaces of the enzymes. At this moment the substrates may come into contact with their specific enzymes. Chemical reactions, by which certain physiologic processes are released, may take place. The role of lipides in the living cell is similar to the role of lipide-soluble narcotics in the experiments of Warburg and co-workers (154). These authors demonstrated that oxalic acid and amino acids are easily oxidized on the surface of charcoal. This oxidation is inhibited when the surface of the charcoal is covered

* Several other mechanisms of enzyme inhibition are known. They are not, however, concerned with the irritability of the cell.

by molecules of various narcotics which prevent the molecules of the above-mentioned acids from coming into contact with the charcoal.

It has been demonstrated by Heilbrunn (59) and his students that, in a great variety of cells, stimulation is always accompanied by the release of calcium. Moreover, Barth (4) has advanced the view that calcium is bound to the lipoproteins of the cellular cortex. There is some evidence that within the chromidia the phosphoric acid parts of the phosphatides and of other compounds, particularly ribonucleic acid and respiratory coenzymes, are kept together by means of the bivalent cations calcium and magnesium. The carboxyl groups of proteins and the phosphoric acid parts of phosphatides may also be linked together by means of the above-mentioned bivalent cations. In this connection it is of interest to note that adenosinetriphosphatase is a calcium-activated enzyme (for literature, see 117) which is intimately associated with myofibrils and cytoplasmic fibrils. It is assumed that upon stimulation the lipides are temporarily separated from the proteins. The linkages between the phosphatides, calcium, and nucleoproteins are broken down. The calcium liberated upon stimulation must activate adenosinetriphosphatase and induce the complicated chain of chemical reactions underlying phosphorylation degradation of glycogen, glycolysis, and finally aerobic oxidation (respiration). Energy, necessary for the continual reconstitution of the cytoplasmic fibrils and for the folding or unfolding of their polypeptide chains, is released. The folding and unfolding of the polypeptide chains of various fibrils is the essential phenomenon of a great variety of physiologic functions induced upon stimulation.

It is supposed that the lipid films covering the surfaces of enzymes are continuously and very rapidly broken down and reconstituted again in a certain rhythm. These rhythmic changes, which go on even in resting cells, are strongly accelerated upon stimulation. Consequently it must be assumed that the activity of the enzymes and, in general, metabolism also proceed in a certain rhythm. Various substances of physiologic importance may be bound and released in a rapid rhythm. Tetanic muscle contractions and nerve conduction are good examples of physiologic functions proceeding in a rapid rhythm. No doubt organisms exist which exhibit either a rapid or a slow metabolic rhythm.

Nerve conduction is only a specialized form of the general conductivity of protoplasm. This has been emphasized particularly by Lillie

(80). Chromidia, which exhibit irritability, are concatenated with each other by means of interchromidia. Therefore the metabolic phenomena that are displayed by chromidia may influence each other. The changes which occur upon stimulation are transmitted from one chromidium to the next, and so on. Conductivity, which is intimately associated with fibrillar structure, is a general property exhibited by all kinds of living fibrils. It seems that general contractility and general conductivity are essentially the same phenomenon. Some facts indicate that not only lipides but also proteins are concerned with conductivity. Changes of the lipides and changes of the cytoplasmic polypeptide chains are probably intimately associated with each other. It is supposed that upon stimulation the lipides are temporarily separated from the proteins of the cytoplasmic fibrils, and that in this moment the degree of folding of the polypeptide chains and the degree of hydration are altered. (Upon stimulation numerous varicosities appear on the thread-like pseudopodia of various rhizopods (59, page 540). It is plainly evidenced by this fact that hydration of the cytoplasm is altered upon stimulation.) This folding and hydration must return to the previous state when the lipides are reattached to the proteins. This change of the lipides and the polypeptide chains may be rhythmically transmitted along the cytoplasmic fibrils. This hypothesis is supported by the facts cited below.

Bethe (8) has demonstrated that the protoplasm of the nerves becomes acid upon stimulation. This acidity may be attributed to the nucleic acid (137). Large amounts of ribonucleic acid are known to occur within the cytoplasm of all neurons. Hydén (73) found that ribonucleic acid, which is concerned with protein synthesis, partially disappears when nerves are stimulated, until the animals used for the experiments are completely exhausted. This proves that not only lipides, but also proteins, play an important role during nerve conduction. Probably the polypeptide chains of the cytoplasmic fibrils are folded and stretched during nerve conduction. Disappearance and subsequent reappearance of ribonucleic acid prove that proteins are broken down upon excessive stimulation of the nerves and resynthesized again upon recovery. It is probable that, in nerves stimulated until the animals are exhausted, the polypeptide chains are so rapidly folded and unfolded that they break, the broken polypeptide chains being easily attacked by proteolytic

enzymes. Proteins are disintegrated probably because the lipides have been previously broken down. This is supported by the fact that the mass of the Golgi apparatus which is known to contain large amounts of lipides is strongly decreased in injured neurons (for literature, see 30,64). No doubt, in strongly stimulated cells the breakdown and reconstitution of the living substance are strongly accelerated.

Thus, it can be inferred from the above-mentioned facts that upon stimulation the nucleic-acid-calcium-lipide compounds of the nerves are broken down and reconstituted, this change proceeding along the fibrils with great rapidity in a certain rhythm. This must, of course, be accompanied by the temporary appearance of negative and positive electrical charges on the fibrils. Moreover, bioelectric currents have to be generated. The breakdown of the nucleoprotein-calcium-lipide compounds is probably accompanied by the temporary folding of the polypeptide chains. These chains must be stretched when the above-mentioned compounds are reconstituted. Thus, it seems that during nerve conduction lipid films are broken down and reconstituted; this process may be accompanied by very short folding waves of polypeptide chains moving rapidly along the fibrils (103). Permeability is always increased upon stimulation (65,66). Lipide-insoluble substances may penetrate the cell surface. The mechanism of these phenomena is discussed in the next section.

Nerve conduction is generally compared to successive explosions occurring along a series of piles of gunpowder (for literature, see 59). It is governed by the all-or-none law. Periodicity in the structure of fibrils is the prerequisite for this kind of transmission. The periodic structure of the cytoplasmic fibrils is proved. These fibrils consist of chromidia concatenated with each other by means of interchromidia. Energy from various organic compounds is released by the enzymes which are accumulated within the chromidia and therefore the latter are comparable to gunpowder piles. Periodicity due to nodes of Ranvier (59) may be of secondary importance for nerve conduction. When an "explosion" is released within a certain chromidium the polypeptide chains of the adjoining interchromidium may be folded, thus causing the next chromidium to explode, and so on. It is certain that nerve conduction is not a disturbance which gradually fades from the stimulated point. This kind of transmission would be possible only in fibrils that have an unperiodic continuous structure.

E. PERMEABILITY

Permeability is the capacity of the plasma membrane to allow the transit of relatively small (noncolloidal) molecules from the surrounding medium into the interior of the cell and vice versa. There are two kinds of permeability phenomena entirely different in their nature: passive penetration and active transfer (for literature, see 66). Energy derived from cellular metabolism is necessary for the active transfer of molecules across the plasma membrane or across the whole cytoplasm. In contrast to passive penetration, active transfer may be inhibited by narcotics. Passive penetration occurs without any expenditure of energy. The passage of lipid-insoluble substances through the plasma membrane is chiefly due to active transfer. Only lipid-soluble substances and, to a certain extent, some lipid-insoluble substances of low molecular weight are able to penetrate the cell passively.

Lipides, which are present within the whole cytoplasm, are strongly concentrated within the cortex (see page 19). This fact accounts for the quite general and well-known phenomenon, first clearly recognized by Meyer and Overton (see 66), that lipid-soluble substances easily penetrate the cytoplasm and accumulate there without any expenditure of energy. Also some lipid-insoluble ions may, to a certain extent, penetrate the cell without metabolic intervention, *e.g.*, when the Donnan equilibrium is brought about. Passive penetration is best explained by the lipid-sieve theory of Collander and Bärilund (for literature, see 66). The plasma membrane is regarded as a lipid film pierced by numerous holes, which allow, to a minor extent, the passage of some lipid-insoluble substances.

Energy is required for active transfer. There is some evidence that this energy is released chiefly by carbohydrate combustion (66). Many substances, indispensable to life (amino acids, mineral salts, carbohydrates, etc.), are lipid insoluble and therefore their uptake is entirely due to active transfer. The active transfer of lipid-insoluble substances proceeds most rapidly in growing, resorbing, excreting, and secreting cells. The mechanism by which the active transport is brought about is not known. Nevertheless, in this article an attempt is made to construct a hypothesis accounting for the phenomena observed and based on investigations dealing with the structure of the cytoplasm. Active transfer may be best explained by a dynamic lipid-sieve theory.

Investigations dealing with the structure of the superficial cytoplasmic layer have been described on pages 17-20. In this section an attempt is made to explain active transfer by regular and reversible structural alterations of the plasma membrane induced upon stimulation. It is obvious that the free penetration of lipid-insoluble substances is prevented by the lipides present within the plasma membrane. Lipide-insoluble substances may penetrate the plasma membrane only where lipid molecules are absent. Bernstein and Höber (65) explained the increase of permeability upon stimulation by assuming that temporary holes appear within the plasma membrane. Thus it may be supposed that upon stimulation the lipid-protein compounds of the plasma membrane are broken down and reconstituted again very rapidly in a certain rhythm, this change proceeding like a wave from the stimulated point. The meshes between the protein fibrils represent the pores of the plasma membrane. The lipid molecules oriented perpendicularly to the polypeptide chains prevent the lipid-insoluble substances from penetrating the pores. The lipid-insoluble substances may pass through the plasma membrane only in the places which, upon stimulation, are temporarily deprived of their lipides. Thus, it is assumed that the "doors" for the penetration of the lipid-insoluble substances are rhythmically opened and closed.

The cortex and the plasma membrane are constructed of fibrils which probably consist of nucleic-acid-containing and nucleic-acid-free sections regularly alternating with each other. Enzymes of the energy-yielding activities are supposed to be chiefly associated with sections which contain nucleic acid. The rod-shaped lipid molecules are oriented perpendicular to the polypeptide chains of these fibrils. Energy, necessary for the active transfer of lipid-insoluble substances, must be released when, upon stimulation, the nucleoprotein-calcium-phosphatide compounds of the plasma membrane are temporarily broken down. Under the influence of this energy a folding of the polypeptide chains of the fibrils constituting the plasma membrane may be induced. The polypeptide chains may be folded when the nucleoprotein-calcium-phosphatide compounds are temporarily broken down and they may be stretched when the above-mentioned compounds are reconstituted. A rhythmical increase and decrease of the size of the pores of the plasma membrane may be brought about by the folding and unfolding of the polypeptide

chains. Consequently various lipide-insoluble substances may be forced in and out of the cells. Thus, the general contractility of the fibrillar components of the cytoplasm may explain the active transfer of lipide-insoluble substances (104). The enchylema, similar to blood in capillaries, is subjected to a continuously changing hydrostatic pressure, because the cytoplasmic texture is condensed in some regions of the cell and released in others. The colloidal osmotic pressure (46a) of the enchylema may be due chiefly to albumins. Lipide-insoluble substances must be resorbed wherever, beneath the cell surface, the hydrostatic pressure is less than the colloid osmotic pressure and they must be excreted wherever the reverse occurs.

There is strong evidence that lipides, particularly phosphatides, play the role of an isolating factor within the cytoplasm. They control the enzymic activity and they neutralize, in association with other substances, the electrical charges of colloidal particles. The electrical charges of the fibrils of the plasma membrane are strongly reduced when the phosphoric acid parts of the phosphatides and nucleic acids are kept together by means of bivalent cations, particularly calcium. Positive (calcium ions) and negative (phosphoric acid) electrical charges must appear when, upon stimulation, the nucleoprotein-calcium-phosphatide compounds are temporarily broken down. These electrical charges may be important for the simultaneous absorption and active transfer of anions and cations. Water is bound by the folded polypeptide chains when the fibrils are induced to contract. Thus, anions, cations, water, and lipide-insoluble nonelectrolytes may be bound by the fibrils of the plasma membrane when, upon stimulation, the nucleoprotein-calcium-phosphatide compounds are temporarily broken down. These substances must be released when the above-mentioned compounds are reconstituted.

Lipide-insoluble substances which pass through plasma membrane are transported into the interior of the cell. These substances may be carried away by currents within the enchylema or they may migrate along the cytoplasmic fibrils. In globular cells the cytoplasmic fibrils are oriented in any direction and, therefore, the above-mentioned substances may migrate at random only. In resorbing intestinal cells and in excreting kidney cells the cytoplasmic fibrils are oriented in one direction only. The importance of fibrils for the active transfer of lipide-insoluble substances is plainly evidenced by

this fact. In resorbing cells the lipide-insoluble substances are actively transferred in the direction from the lumen to the base, and in excreting cells in the opposite direction. Heidenhain (58) advanced the view that the water transport of excreting cells may be due to the short contraction waves of the fibrillar components of the cytoplasm. The very short folding waves of the polypeptide chains may proceed within the fibrils. Anions and cations may be bound by these fibrils when positive and negative electrical charges temporarily appear upon the breakdown of the nucleoprotein-calcium-phosphatide compounds. Water is bound by the polypeptide chains which are induced to be folded. All the mentioned substances must be released when the nucleoprotein-calcium-phosphatide compounds are reconstituted and the polypeptide chains stretched. Anions, cations, water, and lipide-insoluble nonelectrolytes may be successively bound and released by the adjoining parts of the cytoplasmic fibrils and by this means transported in a certain direction. Several experimental investigators have arrived at the conclusion that carrier systems must be postulated in order to perceive the mechanism of active transfer (for literature, see 66). The albumins of the enchylema, similar to the albumins of the blood (for literature, see 27a), may adsorb a great variety of lipide-insoluble substances and therefore they may be of great importance for the transfer of these substances across the cell. Short folding waves of cytoplasmic fibrils may induce the enchylema to stream in a certain direction.

It has been suggested that phosphorylation might be the prerequisite for the active transfer of glucose (66). Thus, energy released upon stimulation may be used in order to produce a reversible chemical change by which the penetration of lipide-insoluble substances is highly facilitated.

F. DEFENSE MECHANISMS

Any injury acts like a stimulus which increases the metabolic activity of the cell. Energy is provided in order to release a series of apparently purposeful reactions leading to repair of the damage. These apparently purposeful reactions are due to the ordered structure of protoplasm. The cell is able to defend itself against any injurious effect which might be produced by various external agents. If the structure of the protoplasm is profoundly altered under the influence of these injurious agents, the capacity of purposeful reac-

tions is lost. Unpurposeful or detrimental reactions may be called forth. Injury may be produced either by physical or chemical agents.

A harmful effect may be produced only by substances which are able to penetrate the cells. These noxious substances may be either noncolloidal or colloidal (virus, bacterial toxins, etc.).

It is universally accepted that Golgi bodies and mitochondria are able to accumulate a great variety of substances which penetrate the cells (for literature, see 64). Large amounts of substances are passively taken up by any cell. In most cases these substances are not uniformly distributed throughout the cytoplasm, as may easily be demonstrated if the lipide-soluble substances employed for the experiments are dyes. Some of the dyes are selectively accumulated within the mitochondria, others within the Golgi bodies, still others are distributed diffusely, but even in the latter case the respective dyes are more intensely taken up by the mitochondria and the Golgi bodies than by the ground cytoplasm. The distribution of the above-mentioned lipide-soluble substances depends upon their physicochemical properties, and therefore it is possible to predict how they will accumulate within the cytoplasm (92). Golgi bodies and mitochondria have certainly several different functions: one is the accumulation of various rapidly penetrating substances in order to protect the ground cytoplasm against their injurious effect. These substances may exercise a noxious effect on the enzymes present within the cytoplasm and on the structure of this cytoplasm. It is known that the cell is able to detoxify a great variety of poisons by changing their chemical composition. It would be of interest to know whether this process occurs within the Golgi bodies and the mitochondria. Some cells are able to take up colloidal substances to a certain extent. It has been found that these colloidal substances are accumulated within the Golgi bodies and enclosed in vacuoles (for literature, see 64). It would be of interest to investigate whether poisonous colloidal substances (bacteriotoxins) might be rendered harmless by accumulation within the Golgi bodies. Both colloidal and lipide-soluble noncolloidal substances accumulated within the Golgi bodies are enclosed in vacuoles and cast off into the surrounding cytoplasm.

Even substances that are diffusely distributed are subsequently accumulated within vacuoles directly formed within the ground cy-

toplast. The portions of the cytoplasm which are destroyed by the penetrating substances are always separated from the healthy cytoplasm. They are either enclosed in vacuoles or pinched off (24). The Golgi bodies frequently decrease in size or disappear in acutely poisoned cells (see 30,102). Lipides may be released by the Golgi bodies in order to repair the injured ground cytoplasm. It is known that mitochondria are very sensitive to changes in the osmotic pressure of the surrounding medium. Thus, mitochondria may protect the ground cytoplasm by taking up a certain excess of water in case the cells are subjected to the action of hypotonic media. All strongly surface-active substances are known to be powerful cytolyzing agents. It has been demonstrated that these strongly surface-active substances are selectively accumulated within the cellular cortex (102). In contrast to narcotics, the strongly surface-active substances penetrate the interior of the cytoplasm in very small amounts only. Extraneous coats covering the plasma membrane of any cell may protect the cytoplasm against the detrimental effect of the above-mentioned strongly surface-active substances.

Large amounts of lipide are present within the nuclei (152), whose membranes are freely permeable to a great variety of substances (24,90). In spite of these facts, the nuclei remain, as a rule, unstained when the cells are immersed in solutions of lipide-soluble vital dyes. The cytoplasm only is distinctly colored. The possible explanation may be the insolubility of these dyes in the nuclear lipides. Stoneburg (152) found that there exist great differences between cytoplasmic and nuclear lipides. The former consist of phosphatides with unsaturated fatty acids in their molecules, and the latter of phosphatides with saturated fatty acids only. Moreover, the amount of cholesterol is much larger within the nucleus than within the cytoplasm.* The usual vital stains are all probably soluble only in phosphatides which contain unsaturated fatty acids in their molecules. At any rate, it has been found that this holds true for two of these dyes, namely, Nile-blue sulfate and Sudan III (74). Thus, it is possible that the nucleus is protected by the different com-

* From this fact it must be concluded that the function of the nucleus may be influenced selectively only by substances which are weakly soluble in phosphatides with unsaturated fatty acids and cholesterol, but strongly soluble in phosphatides with saturated fatty acids and cholesterol. Only the latter substances may be strongly accumulated within the nucleus. It is possible that mutations may be experimentally produced only by these substances.

position of its lipides against the penetration of various poisonous substances. This may be of great importance for the function of the nucleus (see page 62).

Foreign proteins, particularly bacteriotoxins and viruses, are known to be powerful cell poisons. All kinds of cells may be cytolized under the influence of these substances. Evidence has been presented that some of these substances are lecithinases (145). The latter are able to produce cytolysis by disintegrating the lipide-protein compounds of the affected cells (111,129). Nevertheless, the cells are able to defend themselves against these colloidal poisons. A great variety of proteins may act as antigens by calling forth the production of antibodies (for literature, see 12,37,145). The poisonous effect of the antigens is neutralized in some way by the antibodies. The latter are also proteins, more accurately expressed, globulins. Probably any cell is able to produce antibodies to a small extent, at least intracellular antibodies against virus infection. Viruses are known to be intracellular parasites. Nevertheless, certain cells have specialized themselves in the production of large amounts of antibodies which are given off into the blood. Strong evidence is accumulating that in the case of vertebrates the cells of the reticulo-endothelial system (macrophages) are concerned with the production of antibodies. These cells are known to engulf various colloids, also foreign proteins which may be present within the blood. Moreover, evidence has been provided that the above-mentioned cells are implicated in the synthesis of the blood plasma proteins, including globulins. In this connection it is of interest to note that antibodies have been recognized as being merely modified plasma globulins. Sabin (133), injected into rabbits a dye-protein known to be an antigen and traced in various cells the distribution and fate of this colored compound. She found that the dye-protein is accumulated only within the macrophages, and that antibodies against the above-mentioned colored compound are produced coincident with the time when the shedding of the superficial parts of the cytoplasm of these macrophages is strongly accelerated. It has been inferred that both normal and antibody globulins originate from the part of the cytoplasm of the macrophages which is carried out into the blood.

Antibodies are proteins. The production of antibodies means increased protein synthesis within the macrophages. The phenomena associated with increased protein synthesis in cells other than the

above-mentioned have been described by Caspersson and collaborators (for literature, see 42). It is inferred from these investigations that the amount of ribonucleic acid present within the nucleolus and the cytoplasm is greatly increased in the macrophages which are induced to synthesize antibodies. Antibodies are elaborated within the cytoplasm. Cytoplasm is constructed of fibrils which consist of ribonucleic-acid-containing chromidia and ribonucleic-acid-free interchromidia regularly alternating with each other. Caspersson (18) presented some facts indicating that the interchromomeres of the chromosomes consist of proteins of the globulin type. The same may hold true for the interchromidia. Protein synthesis is accelerated when the amount of ribonucleic acid present within the chromidia is increased. Interchromidia should be regarded as the proteins which are elaborated by chromidia. It is supposed that both normal blood proteins and antibodies are synthesized by the chromidia of the cells of the reticuloendothelial system. Consequently it must be assumed that interchromidia represent the site where the newly formed normal blood proteins and antibodies appear.

Several hypotheses have been advanced to explain the specific reaction between antigens and antibodies (for literature, see 12,145). The most satisfactory hypothesis is that there exists, to some degree, a stereochemical correspondence between antigens and antibodies. Antibodies are produced when protein synthesis is modified under the influence of antigens previously taken up by the cells. The protein synthesis of the chromidia may be modified only when contact between the molecules of the antigens and the globulins of the interchromidia is established. The structural adaptation between antigens and antibodies may be gradually brought about when the latter are synthesized within the interchromidia. The role of the Golgi apparatus and of the mitochondria during the formation of antibodies is not known. Probably the amount of both cytoplasmic components is greatly increased, which always occurs when cell activity is augmented.

G. ANABOLIC ACTIVITY

Many life phenomena can proceed independently of the nucleus. This is evidenced by the numerous investigations performed on non-nucleated cells (erythrocytes of mammals) and on cytoplasmic frag-

ments experimentally deprived of their nuclei (for literature, see 13,55,153). In general it may be stated that catabolism and all life phenomena which are dependent upon it may be displayed by cytoplasmic fragments deprived of their nuclei. Catabolism, particularly respiration and fermentation, is exhibited even by minute cytoplasmic fragments of minced tissues. Irritability, nerve conduction, selective permeability, active transfer of lipide-insoluble substances, active contractility, cytoplasmic streamings, ciliary and amoeboid movements, etc., are independent of the nucleus. Even anabolic activity may proceed, to some extent, in the absence of the nucleus. Thus, synthesis of carbohydrates (carbon assimilation) and fats occur within cytoplasmic fragments which contain chloroplasts, but which are deprived of their nuclei. It would be of interest to know whether the cytoplasm is also able to synthesize some unspecific oligopeptides, independently of the nucleus. Dipeptidases which are present within the cytoplasm in large amounts (Linderstrøm-Lang, Holter, Kopac, Duspiva, Brachet; for literature, see 13) may play some role in these synthetic processes. Nevertheless, it is certain that the synthesis of cell-, organ-, and species-specific proteins cannot be accomplished without the participation of the nucleus. This may also hold true for a variety of other substances. Embryologic development is a magnificent process of chemical syntheses directed by the nucleus. Therefore reproduction, growth, and differentiation are impossible without the nucleus.

The nucleus is the site of hereditary factors. The cytoplasm is relatively unimportant for heredity. Nevertheless, it is not unimportant for the anabolic activity of the cell. In this respect the cytoplasm is no less important than the nucleus. The cytoplasm cannot be regarded merely as a product of the synthetic activity of the nucleus. The growth of the cell is the result of mutual interaction between all components of the nucleus and of the cytoplasm. This is plainly evidenced by the fact that the nucleus which is deprived of its cytoplasm does not grow and does not perpetuate itself; this is also true of the cytoplasm which is deprived of its nucleus. Evidently, the cytoplasm elaborates substances which are indispensable to the synthetic activity of the nucleus and vice versa. The substances elaborated by the cytoplasm, independently of the nucleus, are probably simple and nonspecific. This may be the reason why the cytoplasm, although indispensable to anabolism, is relatively unimportant for

heredity. The nucleus is of paramount importance for heredity, probably because the substances synthesized within the cytoplasm under its direction are complicated and highly cell- and species-specific. This specificity associated with an amazing variability is exhibited almost exclusively by proteins; the specificity of carbohydrates and fats being rather unimportant. Moreover, it is known that various regulatory substances, such as hormones, vitamins, coenzymes, etc., are not species specific. Thus, the nucleus is the site of hereditary factors because it directs the synthesis of the highly specific proteins. The specificity of life phenomena is due to the nucleus. It may be that nonspecific oligopeptides, independently elaborated by the cytoplasm, are synthesized into highly specific proteins under the influence of the nucleus. The above-mentioned oligopeptides may be necessary for the activity of the nucleus. Thus, the chief function of the nucleus is the direction of protein synthesis (13,19) and the elaboration of substances necessary for this activity. Protein synthesis is controlled qualitatively by the nucleus alone. Nevertheless, there is some evidence that protein synthesis is controlled quantitatively, not only by the nucleus, but also by the cytoplasm.

Protein molecules are highly complicated structures and therefore notions universally employed in comparative anatomy may also be of importance for protein chemistry. In comparative anatomy it is customary to distinguish between homologous and analogous organs. The former are of common origin but may be very different in function. The latter have the same function but are of different origin. Proteins may be classified in a similar way. According to their physiologic role the proteins may be classified into: self-perpetuating proteins (nucleoproteins), enzymes, scleroproteins, contractile proteins, reserve proteins (yolk, etc.), protecting proteins (mucilages), defense proteins (antibodies), etc. The proteins of each group are analogous to each other because they have the same function. However, they are not identical with each other; they are species specific, they differ in the structure of their molecules and they are widely different in origin. The species specificity of proteins gradually arose during phylogenetic development. According to their origin the proteins may be classified into the proteins of protozoans, coelenterates, annelids, molluscs, vertebrates, etc. Proteins of common origin are homologous, although their physiologic role may be very

different. No doubt the structure of protein molecules is the expression both of their function and of their phylogenetic origin.

The functional differentiation of proteins takes place during embryologic development. A great variety of proteins, very different in functions, are gradually elaborated. These proteins are species specific, like the proteins of the chromosomes and of the cytoplasm of the still undifferentiated eggs (see 29). Preformation and epigenesis are two hypotheses with which not only embryology but also protein chemistry are concerned (13). The question arises whether all specific proteins are preformed within the egg and merely reproduced, or whether they are gradually synthesized during the development. An intermediate hypothesis seems to be most probable. Specific nucleoprotamines and nucleohistones preformed within the nucleus may represent the starting point in the synthesis of specific and more complicated proteins (18,75). It is universally accepted that embryologic development is a recapitulation of phylogenetic development. Therefore it is very probable that protein synthesis during the ontogenetic development is also a recapitulation of protein synthesis during phylogenetic development. Needham (110) may have had a similar idea when he wrote: "For both age and phylogenetic status involve increased complexity and hence increased specificity, of protein molecules as of morphological organization."

The mechanism by which protein synthesis is brought about within the cell has been disclosed by the investigations of Caspersson, Schultz, Hydén, Brachet, *et al.* (for literature, see 13,42). Only proteins associated with some kind of nucleic acid are able to increase their own substance and to reproduce themselves. Thus, nucleic acid must be concerned with protein synthesis. Spiegelmann (147) has advanced the view that the nucleic acid may be the necessary source of energy for protein synthesis. Ribonucleic acid, elaborated by the heterochromatin, is stored within the nucleoli and later transferred in some way into the cytoplasm. The amount of ribonucleic acid in the cytoplasm is increased and therefore protein synthesis is induced. No doubt this ribonucleic acid is taken up by the chromidia (and comparable components), which start to produce proteins.

Protein synthesis is induced by the above-mentioned mechanism. Nevertheless, another mechanism must also be present by which protein synthesis is depressed, controlled, and kept on a suitable level.

There is strong evidence that not only the catabolic but also the anabolic activity of the cell is controlled by means of lipides and similar substances. It is possible that the described mechanism of protein synthesis is inhibited by lipides which are elaborated within the cytoplasm and which penetrate the nucleus. Energy is required for the synthesis of living substance. Lipides control the enzymes concerned with the energy-yielding activities and therefore any change in the amount or the condition of the lipides may either increase or decrease anabolic activity. Moreover, it is possible that lipides also control this anabolic activity by separating the nucleoproteins from the materials necessary for chemical syntheses. Thus, protein synthesis may be the result of a compromise between two antagonistic stimulating and inhibiting agents. Protein synthesis induced and accelerated by nucleic acid may be depressed by lipides and thereby brought to a suitable level. The lipide content is increased in tissues, particularly muscles, whose activity is strongly intensified. The lipide content drops, however, to the original level when the tissues hypertrophy as a result of increased work (for literature, see 10, pages 250-257). This fact indicates that protein synthesis is brought about when the lipides of the cell are subjected to some change. Probably protein synthesis is induced in the above-mentioned strongly stimulated tissues because the lipides are broken down more rapidly than they are reconstituted.

It has been stressed by several investigators (for literature, see 64), particularly Malaczyńska-Suchcitz (86) that Golgi bodies and mitochondria may play an important role in the processes of growth and chemical syntheses not necessarily concerned with the elaboration of secretion products. There is strong evidence that mitochondria and Golgi bodies are centers of lipide synthesis. It is probable that phosphatides are chiefly elaborated by Golgi bodies and cholesterol by mitochondria (104). Several investigators, particularly Parat (112), observed that "diffuse lipoids" are present in the vicinity of the Golgi apparatus. This may indicate that lipides synthesized by this cellular organ, are released, and diffuse out into the surrounding cytoplasm. It is supposed that the lipides elaborated by Golgi bodies and by mitochondria are taken up by the fibrillar constituents of both cytoplasm and nucleus (104). Within the nucleus the cytoplasmic lipides must be transformed into the specific nuclear lipides. Only phosphatides with saturated fatty acids in their molecules were found

to be present within the nucleus of various cells (152). In contrast to the nucleus, the cytoplasm contains large amounts of unsaturated phosphatides (see page 53). Consequently it must be assumed that the cytoplasmic lipides are saturated after their penetration into the nucleus. Thus, it seems that protein synthesis is controlled quantitatively by the heterochromatin and the nucleoli present within the nucleus, and by the Golgi bodies and the mitochondria present within the cytoplasm.

The specificity of life phenomena is chiefly due to proteins. This specificity is determined and inherited by genes which are transmitted from one generation to the other. Consequently it must be assumed that protein synthesis is qualitatively controlled by active genes present within the euchromatin and almost entirely absent within the heterochromatin. Under the influence of these genes the specific proteins are synthesized. Thus, heterochromatin seems to be concerned with the quantitative control and euchromatin with the qualitative control of protein synthesis.

Embryologic development is the result of interaction between genes present within the nucleus and chromidia (and comparable components) present within the cytoplasm (13). Embryologic differentiation cannot be due to any permanent change of the chromosomes. This is evidenced by the fact that all cells of the embryo contain, as a rule, the same chromosome set. Embryologic differentiation is due to changes produced within the cytoplasm under the influence of genes. Some of these changes are permanent. It may be that during embryologic development the chromidia mutate under the influence of the genes. Thus, embryologic (particularly histologic) differentiation may be, at least partially, the result of differential mutations of the chromidia. The fate of cells and organs during development may be determined by these chromidial mutations. This hypothesis accounts for the fact that cells may be temporarily dedifferentiated without losing their specific character. This phenomenon occurs during mitosis and in tissue cultures. The specific cellular components and the specific proteins characteristic of these components are completely broken down. Only identical components are reconstituted again. These components are elaborated by the chromidia (see page 63) and therefore it must be assumed that determination is due to a permanent change in the chromidia.

Differentiated cells are temporarily dedifferentiated before any cell division takes place. In normal growing tissues the specific cellular structures are broken down before cell division and reconstituted completely afterward. In cancer tissues specific cellular structures are also broken down before cell division but they are not at all, or only incompletely, reconstituted afterward. This proves that in cancerous tissues synthetic processes, particularly protein synthesis, are not only strongly intensified (21) but are also qualitatively changed. It is obvious that in cancer tissues the capacity of reconstituting the specific cellular components and of resynthesizing the specific proteins of these components is either strongly impaired or completely lost. It may be that cancer is due to some specific mutation of the chromidia. Graffi (50) demonstrated that cancerogenic substances are accumulated within the ground cytoplasm where the chromidia are present. It has been emphasized by Loeb (84) that cancer is due to a permanent change of the cytoplasm similar to the changes which occur during embryologic differentiation. Moreover, Haddow (53) and Darlington (33) advanced the view that mutations of cytoplasmic corpuscles (plasmagenes) may be the chief cause of cancer.

Various substances elaborated by the genes may induce the chromidia to mutate. The genes correspond to the chromomeres which contain proteins and thymonucleic acid in association with lipides (52). Moreover, it must be assumed that various enzymes are present there. This is evidenced by the fact that thymonucleoproteins and lipides within the nucleus are continuously broken down and reconstituted (62), which indicates that even the substance of the genes is continuously broken down and reconstituted. Any gene must have its specific metabolism. Genes act in different ways, probably because they produce different substances which penetrate the cytoplasm and change its activity. These substances may be degradation products or derivatives of proteins, amino acids, phosphatides, sterols, nucleic acids, etc. The importance of mononucleotides for embryologic differentiation is particularly stressed by Brachet (13). Sex hormones may be regarded as the end products of sterol metabolism of certain genes. In this connection it is of interest to note that cholesterol is present within the nucleus (152). The substances elaborated by the genes may be taken up by the chromidia. The latter may mutate when this occurs. A change in the anabolic and

catabolic activity of the chromidia may result. Various new compounds, particularly proteins and biochemical agents (coenzymes, hormones, etc.), may be synthesized. Simultaneously new morphologic entities of the cytoplasm may be constructed.

As already stated above, the composition of nuclear and of cytoplasmic lipides differs greatly (152). This may facilitate the rapid diffusion of the lipide-soluble substances elaborated by the genes from the nucleus into the cytoplasm, and simultaneously prevent the penetration in opposite direction of similar substances eventually elaborated by the chromidia. This may be of great importance for the function of the nucleus, particularly for preventing the occurrence of somatic mutations of genes during embryologic development and in general for keeping the mutation rate low. It is known that a high mutation rate is very dangerous to multicellular organisms, but not at all to microbes, which have a tremendous proliferating power. Runnström (private communication) advanced the view that degradation products of lipides, similar in their action to detergents, might be the substances which occasionally produce gene mutations.

The substances by which embryologic differentiation is brought about operate from within (intrinsic substances) and from without (extrinsic substances). The former do not diffuse out from the cells by which they have been elaborated. The latter diffuse out and act on other cells. This explains the well-known fact that certain regions of the embryo induce alterations in others. Both kinds of substance may produce either permanent changes by causing the chromidia to mutate, or simply inhibition or stimulation of the activity of these chromidia. During embryologic development the action of different genes becomes manifest in different tissues and different organs. Evidently the susceptibility of the cytoplasm to change under the influence of extrinsic and intrinsic substances is gradually developed and differentiated. A change cannot be produced if the cytoplasm is not yet in a reactive state. The competence of the cytoplasm to react to various substances is gradually differentiated. Rapkine and Needham (110) advanced the view that every competence may be characterized by a specific protein. It may be that under the influence of various substances the activity of the chromidia is either permanently (mutations of the chromidia) or temporarily changed; but the chromidia respond only if certain specific proteins have been synthesized by the cytoplasmic fibrils.

Substances which diffuse from certain regions of the embryo may produce changes in others. Quantitative and qualitative differences in the irritability of embryonic cells must be of primary importance for differentiation under the influence of these substances. The selective effect of various drugs is a well-known phenomenon. Differences in the irritability are chiefly due to differences in the condition of the cytoplasmic lipides and lipide-protein compounds. Unfertilized and fertilized sea urchin eggs differ from each other in the condition of their lipides (111,123). This is the probable reason that fertilized and unfertilized eggs react differently to various drugs. Drugs which increase the rate of respiration of the unfertilized eggs may decrease the rate of respiration of the fertilized eggs, even when employed in the same concentration (44,125). For the same reason agents which induce growth in certain regions of the embryo may simultaneously inhibit growth in others. Differential growth is produced. Enzymes, which produce the necessary energy for the synthesis of the protoplasm, may be inhibited in certain regions of the embryo and simultaneously stimulated in others.

The polar and dorsoventral organization of eggs is due to unequal condensation of the cytoplasmic texture (28,98,101,121). This condensation is strongly developed in some cases and weakly manifested in others. The chromidia which are associated with important enzymes are also unequally distributed (ribonucleoprotein gradient, see 13) because they are intimately associated with the cytoplasmic texture. Therefore the blastomeres resulting from cleavage divisions must differ from each other, at least slightly, in the amount of enzymes and cytoplasmic fibrils. These quantitative differences are the starting point of the qualitative differences which arise later (4). In sea urchin eggs, stratified by centrifuging, a gradient in the distribution of the chromidia, which contain enzymes, is produced. The dorsoventral axis of the larvae which develop from these eggs always coincides with the axis of centrifuging (for literature, see 98) and with the experimentally produced chromidia-enzyme gradient (98).

It is probable that during the embryologic development the cytoplasmic fibrils are transformed into the specific fibrils of differentiated cells, such as myofibrils, neurofibrils, epithelial fibrils, etc. This may occur when the chromidia mutate under the influence of genes and when consequently protein synthesis is changed. It may be that

some of the chromidia are transformed into mitochondria and Golgi bodies, as was long ago supposed by Goldschmidt (47,48).

During morphogenesis various protoplasmic structures are not only produced but also broken down. Nucleoli, nuclear membranes, spindles, and other structures may disappear. This is not necessarily due to enzymic breakdown. It is possible that these structures are disintegrated under the influence of substances which are similar in their action to acid detergents and which are temporarily released under certain physiologic conditions. Not only lipides but also proteins are dispersed under the influence of acid detergents (102). Under the influence of similar substances lens-shaped Golgi bodies may be transformed into spheres or fragmented into smaller globules. Erythrocytes are similarly changed under the influence of detergents (131).

The differential adhesiveness of embryonic cells is an important factor in morphogenesis (70,156). The cells adhere to each other by means of their extraneous coats which probably consist of specific proteins. It may be that these cells easily adhere to each other if the proteins of their coats are identical, and that they do not adhere to each other if these proteins are different. Thus, the differential adhesiveness of cells may be the result of divergent protein synthesis occurring during development.

The similarity between memory and heredity has been stressed by several biologists (see 61). In this connection it is of interest to note that the cytoplasm of nerve cells and the chromosomes of all kinds of cells are particularly rich in nucleic acid which is concerned with protein synthesis. Thus, it is possible that synthesis of specific proteins is the essential physical phenomenon paralleling memory, fantasy, and intuition. This hypothesis is supported by the fact that protein synthesis occurs in strongly stimulated neurons (73) and that cells are able to "learn" to synthesize new specific proteins (antibodies, enzymes). Possibly the structure of the proteins constituting the cytoplasmic fibrils is changed during the function of the neurons. Instincts are inherited probably because the specific mode of protein synthesis of the respective neurons is also inherited. It is proved that new mutations arise when the structure of the chromosomes is changed. Moreover, it is possible that the appearance of new ideas is accompanied by changes in the structure of the proteins of the neurons. It may be that the chromidia of the neurons mutate

at any moment a new perception or a new concept arises in the mind. These cytoplasmic mutations may be the cause of a permanent change in the protein synthesis of the brain.

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QUANTITATIVE STUDIES ON COMPLEMENT

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It is happily a rare thing to find in scientific literature a vast body of writings so confusing and so mutually contradictory as those on the manifold activities of complement. Because of the lability of complement (C') and its multiple make-up, experiments conducted under different conditions have often led to exactly opposite conclusions, each correct for the special circumstances involved. Moreover, the methods used in investigations on C' have usually been qualitative rather than quantitative, as judged by the strict criteria of analytical chemistry, and relative rather than absolute, so that results were often not expressed in measures of quantity or activity that had the same

meaning in different laboratories. Small wonder, then, that controversies raged unresolved, and that uncertainty could persist as to whether C' , the weight of which in serum was unknown, was actually a substance or merely a colloidal state of the serum proteins.

No attempt will be made, therefore, in the present review to trace a path through the jungle of the older literature (56), although occasional hardy perennials will be dug up and dissected as the occasion warrants. Discussion will be concerned primarily with two parallel and independent series of investigations which, together, have contributed largely to a more modern and objective, although still woefully incomplete, understanding of the actual nature of complement. One set of studies, that of Ecker and Pillemer and their group was concerned with the isolation, in a state of purity, of the four recognized components of C' . The other, undertaken by the senior writer and his associates, was directed toward the quantitative microestimation of C' in weight units, so that the actual quantity of C' in serum might become known. Other aims were the more accurate determination of the unit of hemolytic activity of C' and more reliable methods for the estimation of the components of C' so that the mechanisms of complement fixation and of hemolysis might be more completely elucidated. Studies dealing with complement as a whole will be considered first.

I. Analytical Microestimation of Complement in Weight Units and Its Consequences

A. METHOD OF ANALYSIS

In the last two decades application has been made in immunology of micromethods for the estimation of antigens and antibodies by techniques which conform to the rigid criteria of analytical chemistry and yield the result in weight units rather than relative titers (27). The use of these methods has resulted in the acquisition of a large body of precise data, notably on the precipitin and agglutinin reactions, the proposal of quantitative theories for these reactions (32), the isolation of analytically pure antibodies (34), and many other practical consequences, such as (36).

An obvious extension of these methods to the study of C' presented difficulties. Because of the accuracy with which specifically precipitable nitrogen could be measured, attempts were made to extend the quantitative precipitin method (33) to the measurement of any

actual uptake of C' in antigen-antibody combination. It was considered that any difference between the amounts of specifically precipitable nitrogen found in the presence of active C' on the one hand, and in the presence of inactivated C' on the other, might serve as a measure, in milligrams nitrogen per milliliter, of C' as defined by Muir (56a), or of the combining components of a more complex C'. Only after many trials was it realized, however, that specific precipitates were capable of combining with far more C', in volume units, than had ordinarily been supposed, and that relatively large volumes of guinea pig serum would be needed if a weight unit for C' were to be established with any degree of accuracy. Details of the method are to be found in the papers of Heidelberger *et al.* (28,39). Accurately measured volumes of antigen and antiserum dilutions in triplicate are separately mixed with saline, with heat-inactivated C' (iC'), and with an accurately measured volume of fresh C'. Control portions are also set up with C' and antigen, C' and antiserum, iC' and antigen, and iC' and antiserum. The immune system in saline serves as an additional control on the completeness of inactivation of the iC'. A typical protocol and result are shown in Table I (28).

TABLE I

ESTIMATION OF C' NITROGEN IN 5.0 MILLILITERS OF GUINEA PIG SERUM (28)

Reagents and results	Number of tubes						
	2	1	1	2	3	3	3
C', ml.	5.0†	5.0					5.0*
iC', ml.			5.0			5.0	
Serum dilution, ml.	1.0†		1.0	1.0	1.0	1.0	1.0
S III‡ dilution, ml.		0.5			1.0	1.0	1.0
Saline, ml.	1†		1	4	4		
N pptd., mg.	0.006	0.012	0.016	0	{ 0.392 0.388 0.386	{ 0.422 0.416 0.426	{ 0.570 0.574 0.578
Mean	0.009		0.016		0.389	0.421	0.574
Subtraction of blank.....					0	0.016	0.009
Specific N pptd., mg.....					0.389	0.405	0.565
Subtraction of iC' series value.....							0.405
C' N pptd., mg.....							0.16

* Hemolytic units left in C' series supernatants, 40; taken, 1250.

† 0.9 of these quantities actually used in the second blank tube. iC' = C' inactivated 50 minutes at 56°C. Type III antipneumococcus rabbit serum was used in 1:10 dilution, containing 0.7 mg. antibody nitrogen per ml.

‡ S III = specific polysaccharide of type III pneumococcus, 0.028 mg. per ml.

It appeared possible, however, that the increased nitrogen might be due, not to the active C' that disappeared from solution during the precipitation, but to an invisible, noncentrifugable nitrogenous component of guinea pig serum which bore no relation to C' and which dissolved irreversibly when the serum was inactivated by heating at 56°C. In order to test this possibility guinea pig serum was filtered through Gradocol membranes of 700 m μ average pore diameter. There was little loss of C' activity. The filtration should, of course, have removed any suspended solid such as the hypothetical substance in question. In spite of this, even more nitrogen was added to a smaller quantity of specific precipitate by the active C' used in this instance (28).

The possibility was also tested that the added nitrogen might not be due to C' itself, but to an easily adsorbable soluble substance capable of adding to any precipitate formed in the solution. Specific precipitates from antipneumococcus horse sera do not fix C' (84), but their general similarity to the rabbit precipitates made it appear possible that any easily adsorbable substance would be removed independently of the C'. Then, if the C' remained, and were specifically bound by an antigen-rabbit antibody precipitate formed in the supernatant any added nitrogen would seem more rigorously due to the C' itself. It was found (28) that practically identical quantities of nitrogen were precipitated by S I (S I = specific polysaccharide of type I pneumococcus; Pn = pneumococcus) from active and inactivated C'-anti-Pn I horse serum mixtures, a behavior in sharp contrast to that of the S III-anti-S III rabbit system. That suitably prepared S I absorbs C' with rabbit anti-S I had already been shown (84a) and this was confirmed with the preparation used. The egg albumin rabbit anti-egg albumin precipitates formed in the second half of the experiment showed a difference between the active and inactivated C' tubes entirely comparable with that in other experiments, so that the nitrogen difference seemed definitely due to the active C' taken up.

The C' and, particularly, the iC' used in the analytical studies were stabilized by storage overnight in the refrigerator and centrifugation before use. In this way appreciable precipitates which often formed, especially in the iC', were avoided, and the total nitrogen precipitated from the iC' tubes, after deduction of the appropriate blanks, was rarely more than 0.01 to 0.02 mg. greater than was precipitated from the immune system dilutions in saline. It seemed reasonable, there-

fore, to ascribe the difference between the C' and iC' values to C' nitrogen. This has been challenged, however, on the ground that iC' adds relatively large and variable amounts of nitrogen to specific precipitates (60). However, in the work of Pillemer *et al.* just cited (60) C' and iC' were not stabilized before use, nor is there evidence that controls were run without immune system in order to correct for any instability. It would also appear from the first table of their paper (60) that the analytical error was at least 10%. It would, therefore, seem justifiable to continue to accept the difference in nitrogen between the C' and iC' series as a measure of C' . It is,

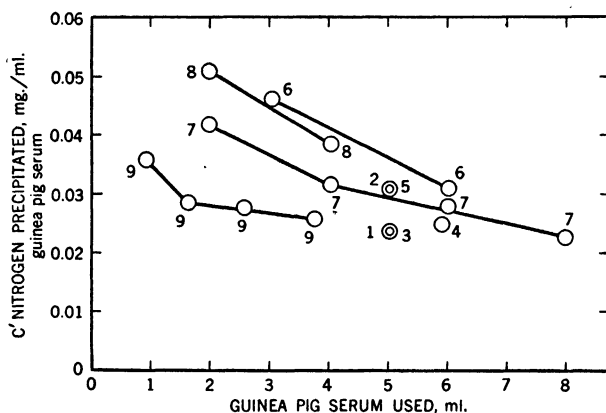


Fig. 1. Complement nitrogen as a function of the volume of guinea pig serum used (28).

however, properly stated (60) that the nitrogen added to specific precipitates may be considered a true measure of C' only if the value is accompanied by data on the extent of uptake of the components of C' . Under the conditions recommended in the quantitative method (28,39), uptake of $C'1$ and $C'4$ was practically complete, usually from 90 to 100%, while that of $C'2$ and $C'3$ varied from 50 to 90% (29, and unpublished results). While the methods used by Pillemer, Heidelberger, and respective co-workers (29,60) for the estimation of the components of C' were faulty, there is agreement that the nitrogen uptake is furnished largely by $C'1$ and $C'4$, so that properly run and properly controlled measurements of the nitrogen contributed to specific precipitates by C' may properly be used as a measure in weight units of C' that is independent of the hemolytic activity.

Although the hemolytic activity of C' may be abolished by heating for five minutes at 56°C., guinea pig sera treated in this way still added about 40% of the usual quantity of nitrogen to specific precipitates. Heating for 45 to 50 minutes was usually necessary to abolish the fixation of nitrogen within the limits of error of the method.

Unfortunately, possession of a method for the measurement of C' nitrogen yielded to specific precipitates by a given volume of guinea pig serum does not immediately permit the calculation of the quantity of C' per milliliter of serum. Comparison of a large number of analyses (Fig. 1) showed that the larger the volume of C' taken, the less was the nitrogen taken up per milliliter. This apparent solubility effect, which will be discussed in connection with C' components, necessitated a rough extrapolation to zero volume and, when this was done, most of the values of C' nitrogen lay between 0.04 and 0.06 mg. per ml. 0.05 mg. nitrogen may therefore be considered as the best available average value, by direct analysis, of C' nitrogen per milliliter of fresh guinea pig serum.

Similar analyses carried out with human (37) and with bovine (3) sera led to comparable values, although the hemolytic titers of these complements are less than that of guinea pig serum. As will be seen later, the lack of parallelism between the values of C' nitrogen and hemolytic titers is due to differences in the spectrum of components in the three complements.

B. ANALYTICAL MICROESTIMATION OF HEMOLYSINS IN WEIGHT UNITS

Hemolysin in antisera has usually been estimated by "titer," hence knowledge of the mode of action of hemolysins on red cells and of the quantitative relations of hemolysins to erythrocytes and C' has been difficult of access. This is not only because of the complicated and sensitive nature of the reactions and their components, but also because methods of assay yielding end "titers" are, taken alone, without significance as to the actual weights of the reactants, since end "titers" are often in opposite ratio to the actual quantities involved. "Titers" depend upon the relative proportions in which the components of a given system interact, as well as upon the absolute quantities present, and are only interpretable, on passing from one reacting system to another, when reacting proportions as well as quantities are known. In the hemolytic system, since only "titers"

of the components could be estimated in an effort to determine the very quantities which would necessarily have to be known before "titers" could be interpreted, lack of theoretical progress in this field is readily understandable.

In the present method quantitative measurements are made of total antibody in hemolytic antisera (40) much as in the quantitative agglutination procedure (30,2,77). Sheep erythrocyte stromata are washed to minimum solubility with saline. Accurately measured volumes of the suspension, in duplicate, are added to the hemolytic

TABLE II

ABSORPTION OF ANTIBODY IN ANTISHEEP CELL HEMOLYSIN BY SHEEP STROMATA
SUSPENSIONS PER 1.0 MILLILITER UNDILUTED SERUM AT 0°C. (40)

Serum	Hemo- lytic titer*	First absorption		Second absorption		Third absorption		Total anti- body N, mg.	Hemo- lytic titer + anti- body N
		Anti- body N, mg.	Hemo- lytic titer	Anti- body N, mg.	Hemo- lytic titer	Anti- body N, mg.	Hemo- lytic titer		
L ₁	1200	0.24	<30	0.05	<4	0		0.29	4100
L ₂	2500	0.11	<120	0	<6			0.11	23000
L ₃ †	3000	0.71	>240	0.11	>240	0	100	0.82	3500
L ₄	2000	0.48	<24	0.03	<12			0.51	3900
S ₁	1200	0.36	130	0.10	36	†		0.46	2600
S ₂	750	0.20	60	0.02	40	0.02	24	0.24	3000
7.60 ₄	600	0.18	<15	0.07	<5	0.02		0.27	2200
7.62 ₄	1400	0.24	15	0.08	<5	0.01		0.33	4200
7.66 ₄	1700	0.29	15	0.09	<5	0.02		0.40	4300
7.83 ₁ **	10	0.09	0	0.01	0	0		0.10	100

* The titrations given are the reciprocals of the dilution at which the sera completely hemolyzed an equal volume of 5% sheep red-cell suspension in the presence of two units of guinea pig complement and are thus independent of the volumes chosen so long as this relation to the red cells is maintained. The antibody nitrogen estimations are calculated to 1.0 ml. of the undiluted serum.

† Pony serum.

‡ Determination lost.

** Rabbit injected subcutaneously with stromata.

sera, diluted with saline, mixed, and allowed to stand 48 hours in the cold. Visible agglutination usually occurs. Blanks, of serum alone and duplicate portions of stromata, are also run. After centrifugation the residues are washed three times with chilled saline and analyzed for nitrogen by a modification of the micro-Kjeldahl method. Analysis of the serum supernatants is repeated until absorption of antibody is complete. A summary of typical data is given in Table II.

Two factors combine to limit the utility of the method for its original object, and both tend to make the results somewhat too high. In spite of thorough washing of the stromata small quantities of soluble material (about 0.04 mg. nitrogen) were invariably found in the washings from the blank tubes. At least a portion of this material gave precipitin reactions in the antisera, but since it is uncertain whether or not similar amounts were washed out of the stromata-antiserum residues no correction has been made for this effect. It is therefore possible that the antibody nitrogen values found are too high by all or a portion of the nitrogen washed out of the controls. Stromata heated after the washing process (46) are more easily centrifuged and appear to be slightly less soluble, but their antibody-binding capacity is too low.

The second limiting factor is that the method, strictly speaking, is one for the estimation not of hemolysin but of total antibody in the hemolytic antisera. Even washed sheep cell stromata are complex collections of antigens and only one or a limited number of these presumably gives rise to true hemolysins. The remaining antibodies stimulated by injection of cells or stromata into rabbits add to the stromata suspension when this is mixed with antiserum or to red cells, but probably take little part in hemolysis. This is probably the chief reason for observed discrepancies in the values given below for the quantity of nitrogen in the hemolytic unit. According to these values the antibody nitrogen figures may exceed true hemolysin nitrogen by a factor of 10 to 100, although the numbers are not to be taken too literally on account of the different units and end points compared. In spite of this, there does appear to be a fair proportionality between total antibody nitrogen content and hemolysin titer in rabbit anti-sheep hemolysin, as already noted (12) on a different numerical basis. If one excludes the serum of the subcutaneously injected rabbit and one other commercial rabbit serum, L₂, that was entirely out of line, ten anti-sheep cell and anti-stromata rabbit sera showed a titer:antibody nitrogen ratio of 3500 ± 600 . In other words, an antiserum which, at a dilution of 1:3500 just completely hemolyzes an equal volume of 5% sheep red cell suspension in the presence of 2 units of C', should contain, within about 20%, 1 mg. antibody nitrogen per ml. None of the sera examined contained as much antibody nitrogen as this in a single milliliter. On this basis it might be reasonable, both from the standpoint of pur-

veyor and purchaser, to sell and buy hemolytic antisera by the milligram of total antibody nitrogen, rather than on the basis of units which might vary considerably from laboratory to laboratory and which provide no inkling of the actual antibody content. Attempts have previously been made to define a weight unit for hemolysin by purification and isolation of the antibody. Locke, Main, and Hirsch (48) purified rabbit anti-sheep cell hemolysin and found 0.007 γ of nitrogen per hemolytic unit in their best preparation. Brunius (9), using the Forssman antigen for selective extraction of the hemolysin, found only 0.0002 γ of nitrogen in a smaller hemolytic unit. In both laboratories the preparations isolated usually contained ten times as much nitrogen per unit. Brunius' value probably represents the actual hemolytic antibody unit more closely than that of Locke, Main, and Hirsch and that now reported, 0.03 γ nitrogen (calculated from the figures in Table II for 0.1 ml. hemolysin dilution). The latter, however, have the advantage of giving an insight into the actual weight of antibody deposited on the red cell from rabbit antisera.

In the use of data obtained by the new method for a consideration of the relation between hemolysin, red cells, and C' in the process of hemolysis it must be remembered that the antibody nitrogen values are maximal and can only be used as hemolysin nitrogen as a first approximation, subject to correction by a factor still to be determined. The total antibody nitrogen is of use, however, for an understanding of the hemolytic process as actually carried out, for all of the antibody combines with the red cells present and also plays a major part in the fixation of C' (40). With values established for C' and hemolysin nitrogen in milligrams per milliliter, several calculations of general interest became possible for the first time. The first of these to be considered will be the relation between C' , hemolysin, and the red cell.

C. RELATION BETWEEN COMPLEMENT, HEMOLYSIN, AND RED CELLS (42)

The rabbit anti-sheep cell hemolysin used in the experiments given in Table III and Table IV contained 0.37 mg. antibody nitrogen per ml. (40). In each instance an equal volume of 1:1250 hemolysin dilution was added to a 5% sheep red cell suspension. 0.2-ml. portions of sensitized cell suspension were used to determine the smallest

amount of guinea pig serum permitting complete hemolysis. The data obtained, including cell counts and derived calculations, are summarized in Tables III and IV.

In the paper of Heidelberger, Weil, and Treffers (42) the molecular weight of hemolysin was taken as 150,000 by analogy with other antibodies in the rabbit (38,75). Since the value of 900,000 now appears more probable (58) the figures in Table III and IV have been corrected accordingly.

As the hemolytic reaction is ordinarily carried out, it is evident that far more C' than hemolysin is present. The latter may be considered largely combined with the red cells in the experiments summarized

TABLE III
PROPORTIONS OF HEMOLYSIN, COMPLEMENT, AND RED CELLS
IN HEMOLYSIS (42)

	Experiment number		
	1	2	3
C' N/ml. guinea pig serum, γ	50	50	50
Fraction of ml. for complete hemolysis of 0.2 ml. sensitized cells	0.004	0.004	0.005
C' N necessary for complete hemolysis of sensitized cells, γ	0.20	0.20	0.25
C' globulin necessary for hemolysis, γ	1.26	1.26	1.58
Molecules of C' necessary for hemolysis*	5.1×10^{12}	5.1×10^{12}	6.4×10^{12}
Hemolysin N combined/0.2 ml. sensitized cell suspension, γ	0.03	0.03	0.03
Hemolysin globulin used for sensitization, γ	0.19	0.19	0.19
Molecules of hemolysin used for sensitization†	1.2×10^{11}	1.2×10^{11}	1.2×10^{11}
Number of sheep red cells/0.2 ml. sensitized suspension	3×10^8	2×10^8	2×10^8
Molecules of C' available for hemolysis of single red cell	17,000	26,000	32,000
Molecules of hemolysin combined with single red cell in sensitization and hemolysis	400	600	600

* The molecular weight of C' is tentatively considered that of C'1, 150,000, as found in the work of Pillemer *et al.* (63).

† The molecular weight of hemolysin produced in the rabbit is approximately 900,000 (58).

in Tables III and IV, since only twice the minimum sensitizing quantity was used, but it seems probable that C' was present in excess. Possibly only an amount equal to the hemolysin, or a small multiple of this amount, entered into actual combination with the sensitized

cells (see 68; also the effect of volume, page 96). Because of the relatively large quantity of C' it is possible that C' as a whole does not function enzymically in hemolysis.

TABLE IV
RELATION OF HEMOLYSIN TO RED CELL SURFACE (42)

Type of ellipsoid and cross section assumed for hemolysin molecule	Cross sectional area of hemolysin molecule, $\text{cm.}^2 \times 10^{10}\dagger$	Total cross sectional area of 400 hemolysin molecules, $\text{cm.}^2 \times 10^{10}$	Red-cell surface* occupied by 400 hemolysin molecules, %	Total cross sectional area of 600 hemolysin molecules, $\text{cm.}^2 \times 10^{10}$	Red-cell surface occupied by 600 hemolysin molecules, %
Prolate, elliptical	0.0088	21	0.4	32	0.7
Prolate, circular	0.0009	2.2	0.04	3.3	0.07
Oblate, elliptical	0.0018	4.3	0.08	6.6	0.13
Oblate, circular	0.0210	50	1.0	77	1.6

* Average sheep cell radius 2.6×10^{-4} cm. (69); calculated by formula 2, reference 67, in simplified form, $\text{area} = 5/12 A^2$, in which $A = 1/2$ cell diameter area of sheep red cell $= 2.8 \times 10^{-8}$ cm.² As has been called to our attention since publication of the original table by Prof. Felix Haurowitz, the preceding calculation is based on a misprint in the paper written by E. Ponder (67). The value for area should be 51×10^{-8} cm.² which strengthens the conclusions of Heidelberger *et al.* (42) as to hemolysis by an additional eighteenfold factor. The new value has been used above.

† Calculated with the aid of the following data: asymmetry factor 1.5, partial volume 0.745, as for other rabbit antibodies (38,75); for prolate cases, ratio of axes 1:10, for oblate cases 1:12 (75, Table IV). The assumption is made that 2400 and 3700 molecules of molecular weight 150,000 would occupy the same area as 400 and 600 of molecular weight 900,000.

The data regarding the antibody molecule (38,75) are such that an uncertainty remains regarding its shape. The experimental value for the frictional coefficient or asymmetry factor permits the calculation of molecular areas on the assumption of either oblate or prolate spheroidal shape. The area occupied by such molecules on the red-cell surface would depend upon their attachment in either the endwise or lateral position. In Table IV calculations of the occupied surface are given for each position of the oblate or prolate spheroidal molecule. Intermediate positions are also conceivable. Depending, then, on which calculation is used, the experimental data indicate that roughly from 0.04 to 1.6% of the sheep cell surface is occupied by hemolysin under the conditions employed. Since two "units" of hemolysin were actually used, complete hemolysis is possible with 0.02 to 0.8% of the cell surface combined with antibody. While these figures should not be taken too literally they do at least indicate by

roughly two powers of ten that sensitization need not involve a coating of the entire red-cell surface. The findings are therefore in accord with the "key spot" theory of Abramson (1) in that only a small portion of the cell surface is involved.

Brunius (9) has calculated the number of the Forssman antibody molecules required to sensitize a single red cell for hemolysis, finding the number as only thirty and the fraction of red-cell surface covered as 0.001%. The area calculations were made on a different basis, but the number of molecules of hemolysin was calculated from a single preparation which showed about ten times the activity per γ nitrogen as four other lots isolated by Brunius, the best preparation of Locke and Hirsch (48), and our own sera, judged by their antibody content. When it is considered that the 400 to 600 molecules of hemolysin indicated in Table III represent double the number necessary for complete hemolysis, and that Brunius used 90% hemolysis as the end point, the agreement among the less active preparations is moderately close. However, sheep red-cell stromata may contain a number of antigens. While the antibodies resulting from the injection of these antigens into rabbits are capable of combining with stromata it does not follow that all of the antibodies actually prepare the cell for hemolysis. The one result used by Brunius for his calculations may therefore be the more significant for the process of hemolysis itself, while the larger number of molecules found in the present experiments represents combination between the red cell and total antibody under the conditions of the hemolytic test as actually carried out with rabbit hemolysin.

The mechanism of hemolysis will be taken up again in another connection.

D. RELATION BETWEEN COMPLEMENT, ANTIGEN, AND ANTIBODY

While the experiments already cited are of limited application in the study of immune hemolysis they furnish a more complete picture of the uptake of C' in certain immune reactions.

It was shown (28) that 40% of C' , by weight, could be added to antigen-antibody precipitates with maintenance of volumes of the reacting components at levels convenient for precise analytical measurement. However, it was scarcely feasible to determine by this procedure the maximum amount of C' capable of reaction with known quantities of antigen and antibody. Instead, the usual technique

of the C' fixation test, with its great delicacy and reproducibility under strictly controlled conditions, appeared capable of affording the necessary information, and could be used to determine at the same time the lower limits of reactivity and the combining proportions of the reacting components. By the use of antigen and antibody solutions of known content and with the aid of the values for C' obtained by Heidelberger (28), the actual quantities of antigen, antibody, and C' could be calculated in weight units for each dilution and mixture.

Details of the experiments have been given elsewhere (42). A typical protocol is shown in Table V.

TABLE V
FIXATION OF COMPLEMENT BY EGG ALBUMIN AND ANTI-EGG ALBUMIN RABBIT SERUM (42)*

Antibody N, γ	0.1 γ egg albumin nitrogen						
	10 \times 10 ⁻³ ml. hemolysin, or 0.05 γ hemolysin N				3 \times 10 ⁻³ ml. hemolysin, or 0.015 γ hemolysin N		
	Complement, ml.				Complement, ml.		
	10 \times 10 ⁻¹ . C'N = 0.5 γ	6.25 \times 10 ⁻¹ . C'N = 0.3 γ	4 \times 10 ⁻¹ . C'N = 0.2 γ	2.5 \times 10 ⁻¹ . C'N = 0.13 γ	10 \times 10 ⁻¹ . C'N = 0.5 γ	6.25 \times 10 ⁻¹ . C'N = 0.3 γ	4 \times 10 ⁻¹ . C'N = 0.2 γ
5	m	0	0	0	0	0	0
3	m	0	0	0	0	0	0
2	0	0	0	0	0	0	0
1	m	0	0	0	0	0	0
0.6	m	0	0	0	0	0	0
0.4	m	0	0	0	0	0	0
0.2	st	0	0	0	0	0	0
0.12	c	sl	0	0	m	0	0
0.08	c	c	sl	0	st	m	m
0.04	c	c	c	sl	c	ac	st
0.024	c	c	c	ac	c	c	ac
0	c	c	c	st	c	c	ac

* Symbols: 0 = no hemolysis; tr = trace; sl = slight; m = moderate; st = strong; ac = almost complete; c = complete hemolysis.

It is evident that in the egg albumin rabbit anti-egg albumin system the smallest quantity of antibody nitrogen detectable with certainty by complete fixation of C' under the conditions used is 0.12 γ and that the antigen-antibody complex is capable of combining with somewhat more than an equal weight of C' nitrogen if the assumptions are made that all C' nitrogen present is taken up when no hemolysis occurs (42) and that 50 γ is the average C' nitrogen content of guinea pig serum (28). Similar results were obtained with specific

polysaccharides of pneumococci and the corresponding antipneumococcus rabbit sera. The final colloidal state of the system failed to influence the proportions in which C' combined, since the quantities of complement nitrogen added to antigen-antibody mixtures yielding specific precipitates with 0.4 to 0.6 mg. nitrogen were of the same order of magnitude as in the clear solutions containing only fractions of a γ of reactants. A chemical, rather than physical, explanation for the fixation of C' is therefore indicated and Ehrlich's concept of C' activity is confirmed in this respect both qualitatively and quantitatively.

At the time Ehrlich's theories were proposed the nature of antigens and antibodies was uncertain and methods of measurement were purely relative. After the introduction of quantitative, absolute methods (27) a large body of precise information regarding immune reactions was assembled, and with the recognition of the protein nature of antibodies it became possible to formulate chemical theories of antigen-antibody reactions which were in accord with modern concepts of the structures of the reacting substances (32). Now that C' has been added to the list of immune substances measurable in weight units it is possible to put these theories to the severe test of their adaptability to the inclusion of C' , hitherto neglected for the sake of simplicity.

It has been shown that the precipitin (32a) and agglutinin (31) reactions might be quantitatively expressed by equations derived from the mass law. Chief among the assumptions made was that both antigen and antibody were multivalent with respect to each other, that is, that each possessed two or more groupings reactive with the other. After the molecular weights of antibodies became known it was possible to assign empirical formulas to specific precipitates formed at certain reference points in the precipitin reaction range (26). Now that data are available on the reacting quantities of C' and the molecular weight of the portion of C' (63) furnishing much of the nitrogen taken up in antigen-antibody combination, it is possible to fit C' into the above quantitative theory.

In papers dealing with this theory, two-dimensional representations of three-dimensional aggregates formed by the union of multivalent antigen with multivalent antibody were depicted somewhat as in Figure 2, in which S represents specific polysaccharide or antigen and A represents antibody and Ea represents egg albumin. A somewhat

similar scheme has been suggested by Marrack (50) and more recently an analogous one by Pauling (59).

The assumption is now made that C' is capable only of loose, easily dissociable combination with dissolved antibody. This is in accord with numerous observations that C' is not fixed by antigen or antibody alone (56). Moreover, it is possible to account for the firm

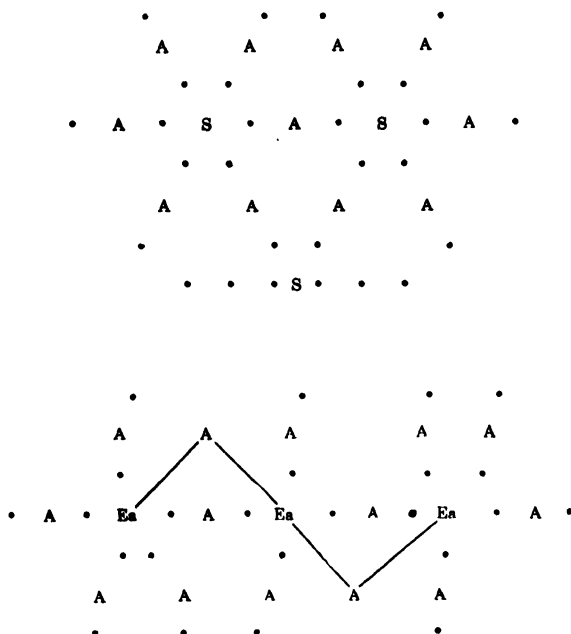


Fig. 2. Two-dimensional representation of three-dimensional specific aggregates (42).

fixation of amounts of C' at least equimolecular with those of antibody by antigen-antibody combinations whether or not these actually separate from solution. In the development of the quantitative theory of the precipitin reaction (32a) it was assumed that multivalent antigen combined with multivalent antibody in a series of competing bimolecular reactions to build up large aggregates like those illustrated, and that the process might be well advanced before the aggregates separated from solution. At high dilutions, especially with rabbit antisera, which form specific precipitates of appreciable

solubility (33b,35), the aggregates would not necessarily separate. Whether or not precipitation occurs, the formation of such multi-molecular aggregates, would bring together and hold myriads of antibody molecules. But by this act any molecules of C' present would be surrounded by antibody molecules. In this way a linkage between C' and A, ordinarily dissociated at once when taking place between single molecules, might become stabilized when occurring between C' and n A. Stabilization might result either through the attraction of contiguous ionized groupings of opposite sign, through hydrogen

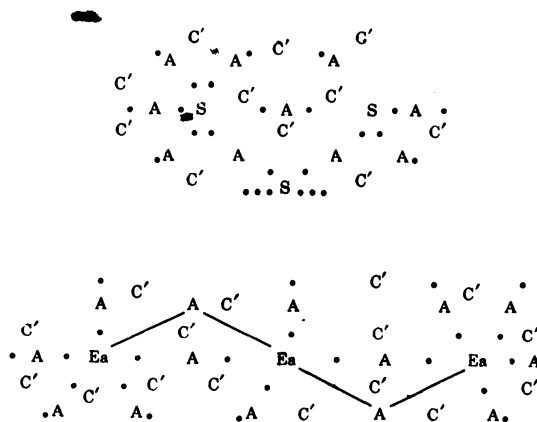


Fig. 3. Two-dimensional representation of uptake of complement by specific aggregates of Figure 2 (56c).

bonding, through spatial accommodation of large groupings on C' and A, or through the presence, on C', as on antigen and antibody, of more than one grouping capable of reacting with A molecules brought into apposition. The result might then be represented in the two-dimensional schemes of Figure 3, corresponding to Figure 2, above. An analogous instance of loose, dissociable combination, capable of conversion into firm union by addition to multivalent antigen-antibody aggregates, is found in the apparently "univalent" antibody which occurs in many immune sera (32a,33b,41 and other papers). This antibody, remaining after fractional absorption of many sera with antigen, forms only easily dissociated, soluble compounds with antigen. However, when multivalent, or complete, antibody is present, the "univalent" or incomplete antibody may add

to the resulting antigen-antibody aggregates in firm, relatively undissociated union and be precipitated just as is the multivalent antibody.

In the above discussion and in the graphic representations C' has been considered, for simplicity's sake, solely in terms of its relation to antibody and without reference to the antigen. However, the data indicate that larger proportions of C' may be added when relatively more C' is present. It is therefore probable that, at least under such conditions, antigen molecules may participate in binding C' firmly in the antigen-antibody aggregate, and it is not excluded that antigen plays some part under all circumstances. It is also possible that antibody may be multivalent with respect to C' , as well as to antigen, so that high ratios of C' to A could be accounted for within the theory on this basis.

Other evidence in accord with the conception of C' fixation as a consequence of aggregate formation by combination of multivalent antigen with multivalent antibody is furnished by the behavior of the pneumococcus type III and type VIII polysaccharide-rabbit antisera reactions, and of the type II and Friedlander type B polysaccharide reactions in rabbit antisera. In each homologous reaction with the specific polysaccharide C' was fixed over a wide range, extending in the type II and type VIII sera to high dilutions at which visible precipitation no longer occurred. However, no visible cross precipitation occurred, even at relatively low dilutions, between S III and anti-type VIII sera, or between S VIII and anti-type III sera, nor was C' fixed in these cross tests. (Recent, as yet unpublished experiments by A. Osler have shown that the small proportion of anti-type III and anti-type VIII rabbit sera which show cross precipitation also fix C' in the cross reactions.) On the other hand S II gave specific precipitation and C' fixation with a Friedlander B antiserum, but the Friedlander B polysaccharide neither precipitated nor fixed C' with type II antipneumococcus rabbit serum. At least in these instances the possibility of multivalent antigen-antibody aggregate formation seems to be a prerequisite for C' fixation even at high dilutions at which the aggregates fail to separate.

It is, however, well known that C' is not fixed by all antigen-antibody reactions which may be expressed in terms of aggregate formation by union of multivalent antigen with multivalent antibody. Although some antigen-horse antibody systems fix C' , pneumococcus

specific polysaccharides react with pneumococcus anticarbohydrate from the horse and with rabbit anticarbohydrate damaged by acid (82) to form aggregates which do not bind C'. The same polysaccharides react with unaltered rabbit and bovine anticarbohydrate with fixation of large amounts of C'. Since almost all antigen-rabbit antibody systems fix C' it would seem possible that rabbit antibody best fulfills the steric requirements for the firm union of C' within the molecular network of the antigen-antibody aggregates. This cannot be entirely due to the equimolecular size of C' and rabbit antibody, since bovine antibody, which also permits C' fixation, is known to have a much greater size (38b). Moreover, hemolysin, the only rabbit antibody known to be of high molecular weight (58), is a classic example of a C'-fixing antibody. Possibly accompanying serum lipides play a part, as was postulated by Goodner and Horsfall (21).

According to these views, then, C' would differ from normal globulin in the possession of one or more groupings capable of forming loose dissociable unions with individual antibody (and perhaps antigen) molecules, but yielding firm, difficultly dissociable combinations, with the exceptions noted, when surrounded by antibody (and perhaps antigen) molecules. In this way the failure of C' to be taken up appreciably by antigen or antibody alone is readily accounted for, also the fixation of C' in qualitatively and quantitatively similar fashion by all but a relatively limited number of antigen-antibody combinations. It is even conceivable that C' would unite with equal firmness with normal globulin if a sufficient number of such molecules could be brought into suitable apposition. The possibility of a firmer union between this protein and C' has been considered as an explanation of the anticomplementary properties of normal γ -globulin (15).

The phenomenon of complement fixation may thus be fitted into the framework of the quantitative precipitin (and agglutinin) theory (32a) with little stretching of postulates which had already shown themselves of some utility. While this affords no proof of the theory it at least justifies use of the theory as a guide for further experiments in a field which the more conventional and alternative theories have failed to clarify.

In the experiments on which the above discussion was based (42) the end point used for estimating the quantities involved was that of complete absence of hemolysis. Under these conditions, in which all

C' is taken up, the immune system is necessarily in excess. It is, however, probable that C' may combine with antigen-antibody systems in multiple proportions, depending upon the relative quantities of the reacting components, just as in the interaction of antigen and antibody (32a). Recent exact measurements of C' activity in which 50% hemolysis is taken as the end point are discussed in the following section. With these methods it has been possible to measure the uptake of C' in large excess by quantities of immune system of the order of 1 or 2 μ g. As had already been noted by Rice (73), at the 50% end point several times as much C' as found by Heidelberger, Weil, and Treffers (42) may be taken up by antigen-antibody combination, especially at 0° (see also 70). This will necessitate some modification of the theory such as was outlined above, but present indications are that the theory still provides the most plausible explanation of C' fixation. The question of C' fixation will again be discussed in Section III.

II. Hemolytic Activity of Complement

A. PROBLEMS OF MEASUREMENT AND CALCULATION

Like many biological processes, the lysis of red cells by C' in the presence of excess antibody is not a stoichiometric process, but is governed by the laws of probability. Thus, if the fraction of cells lysed out of a standard total dose of sensitized erythrocytes is plotted against the amount of C' added, an S-shaped curve is obtained. This curve does not begin at the origin, *i.e.*, a certain initial amount of C' is without lytic effect. As the quantity of C' is increased beyond this range, lysis commences gradually, then accelerates sharply, and finally the slope of the lytic curve diminishes again as complete lysis is approached.

The earliest quantitative measurements of the lytic curve were made by Leschly in 1914 (47). With these data, von Krogh in 1916 (78) proposed an alternation (modified adsorption) formula:

$$x = K \left(\frac{y}{1 - y} \right)^{1/n} \quad (1)$$

in which x is the amount of C' used and y represents the degree of lysis. K is a parameter which varies with the potency of the C' and which actually signifies the amount of C' required for 50% lysis.

The exponent $1/n$ is a constant which determines the shape of the lytic curve.

It was shown by Brooks (7) that the course of the hemolytic curve is largely dependent upon variations in resistance among the cell population. Accordingly, the constant $1/n$ is an expression of the inhomogeneity of erythrocytes with respect to their susceptibility to lysis by C' and antibody. The numerical value of $1/n$ has been found to be about 0.2 in numerous independent experiments widely divergent in time and details of execution (47,52,55). Both Morse (54) and Brooks (8) stressed the fact that complete hemolysis furnishes a rather indefinite end point and stated that determinations in the partial range of lysis, especially at the 50% point, are much more definitive.

While equation 1 fits experimental data well, it must be regarded as an empirical expression since it was derived as an adsorption formula and since the lytic curve owes its shape largely to the heterogeneity of the erythrocytes. It has recently been noted (52) that the probit function (5) can be used to describe the hemolytic process, since if $\log x$ is plotted against the probit of y , a straight line is obtained from which the 50% unit can be calculated. Methods for the calculation of the 50% end point from one or two experimental determinations in the range of partial lysis are based on equation 1. If this is written in logarithmic form:

$$\log x = \log K + \frac{1}{n} \log \frac{y}{1-y}$$

it describes a family of parallel lines of slope $1/n$ and intercept $\log K$. Experimental determination of two or three points in the range of partial lysis can be used to establish the line for a sample of C' . The intercept, $\log K$, of this line is the logarithm of the 50% unit of activity.

If $1/n = 0.2$ is substituted in 1 and if the sample of C' has unit activity in terms of 50% lysis, one obtains:

$$x = \left(\frac{y}{1-y} \right)^{0.2}$$

This function establishes a relation between the degree of lysis and the amount of C' required, on the basis that 50% lysis is brought

about by unit quantity (*i.e.*, 1 ml.) of the C' dilution used. Numerical values for this function have been published by Mayer *et al.* (52) and may be used as factors in the calculation of hemolytic activity from experimental determination of even a single point in the range of partial lysis.

The use of these factors requires precise evaluation of $1/n$, a matter of some difficulty. Careful experimental determinations of this constant under identical conditions vary unaccountably by about $\pm 10\%$. Errors of calculation from this source can be minimized, however, by conducting titrations as close as possible to the 50% point. This difficulty might be circumvented entirely by use of kinetic analyses of hemolytic activity instead of the static measurements customarily employed. Recent, as yet unpublished, determinations of the velocity of immune hemolysis have yielded curves with maximum slopes that vary as a linear function of the concentration of C' and may thus serve as a precise quantitative measure of C' activity requiring no indirect calculations.

Experimental determinations of hemolytic activity by the static method are carried out with 1.0- to 2.0-ml. portions of a suspension of washed sheep erythrocytes standardized to contain 500 million red cells in the volume used and sensitized with a moderate excess of antibody for sheep cells. A carefully measured amount of an analytically precise dilution of active serum (C') is added, the volume is made up to exactly 7.5 ml. and the mixture is incubated at 37°C . for 45 minutes, which permits the lytic reaction to proceed to maximal intensity. (For kinetic analyses, varying short intervals of time are employed, and further lysis is prevented by dilution and chilling of the reaction mixture.) After the incubation period the tubes are centrifuged to remove intact erythrocytes and the supernatant is analyzed spectrophotometrically for hemoglobin. The degree of lysis is calculated by dividing the optical density of the supernatant fluid from a partially lysed analysis by the optical density of the supernatant from an analysis in which complete lysis was achieved by use of an excess of C'. While estimations of C' activity in the partial range of lysis are intrinsically capable of high precision, this goal cannot be attained without careful control of the constituents of the hemolytic system, *i.e.*, cells, sensitizing antibody, and C', and of other factors such as temperature, the concentration of salts, and the volume of the lytic system.

The utilization of a modified Alsever solution (10) for the preservation of sheep blood has made it possible to maintain a stock supply of erythrocytes of reproducible lytic behavior. In two laboratories (45,51) cells stored in this way were found to reach a level of constant lytic response after about four days and to remain in this state for at least ten weeks. This makes it possible to obtain comparable results in tests carried out on different days without correction for a variable response of the cell substrate.

The standardization of the cell suspension used as substrate has generally been carried out by preparing a stated dilution of cells packed by centrifugation. While this procedure may be satisfactory in careful hands, it does not constitute a reliable and universally reproducible method for the dosage of substrate. Although the erythrocyte concentration could perhaps best be defined in terms of the number of cells per unit volume of suspension, this procedure involves the considerable error and labor inherent in counting cells. A simple and yet accurate method of standardization consists in measuring the cell concentration by colorimetric determination of the hemoglobin content (76). The recent application of photoelectric colorimeters has increased the ease and accuracy of these experimental determinations as well as of the measurement of the degree of lysis (45,51,52).

The existence of a reciprocal interdependence between antibody and C' has long been recognized, that is, the more sensitizing antibody present in the lytic system, the less C' is required to lyse a specified amount of cells, and vice versa (43,57). It is, therefore, necessary to define the amount of antibody to be used in determining the activity of C' . This is a rather puzzling problem because there does not exist as yet any certain way of measuring lytic antibody without utilizing C' . While it has been possible to estimate lytic antibody in terms of units of weight the value so obtained is only a rough approximation (pp. 76-79). Many investigators have defined the lytic activity of antibody in terms of the minimal active dose in the presence of an excess of C' , but since the minimal dose depends on how large an excess of C' is present, this procedure is scarcely satisfactory. Probably the best solution consists in utilizing so large an excess of antibody that further changes do not modify the activity of C' (79). Too large an excess, however, must be avoided because this leads to agglutination of the cells and consequent interference with lysis. A recent

quantitative analysis by Kent (44) of the interdependence of antibody and C' in the lytic reaction has yielded the equation:

$$x/y = bx + a$$

in which x represents the amount of antibody, y is the number of 50% units of C' , and a and b are constants. Application of this equation should permit the precise definition of the optimal amount of antibody in determinations of the lytic activity of C' .

Among the factors influencing the lytic activity of C' those involving electrolytes are by far most prominent. First, there is a non-specific ion effect in that the lytic activity varies markedly with the total concentration of salt (52). Although measurements are invariably carried out in approximately isotonic solution, analyses requiring maximal precision must be performed in a medium controlled as to salt content within about $\pm 0.5\%$ of the total, since so slight a change in the molarity of sodium chloride as from 0.145 to 0.151 results in a shift from 143 to 126 50% units of hemolytic activity (52). Second, there is a specific effect of magnesium ion (Mg^{2+}) (52) which has been largely ignored, although noted as early as 1906 (11). The magnitude of the enhancement of lytic activity by minute amounts of magnesium ion (10 to 50 $\mu g.$) is truly striking (Table VI). Reproducible determinations of C' activity therefore necessitate careful control of the concentration of magnesium ion, or, alternatively, the presence of sufficient magnesium ion for optimal activity.

TABLE VI
HEMOLYTIC ACTIVITY IN FIFTY PER CENT UNITS PER MILLILITER OF GUINEA PIG COMPLEMENT WITH VARYING AMOUNTS OF MAGNESIUM ION (52)*

Magnesium ion added, $\mu g.$	Guinea pig complements					
	1	2	3	4	5	6 (pool†)
0	93	84	100	114	98	114
0.09	96	86	109	128	107	114
0.46	110	94	118	138	117	123
2.3	133	131	148	179	134	154
12	173	149	206		168	190
58	203	197			206	240
290					208	231

* The diluent was 0.85% saline buffered at pH 7.3 with 0.005 M phosphate.

† Sera used did not include 1 to 5.

TABLE VII
INHIBITION OF LYSIS BY CITRATE OR PYROPHOSPHATE AND ITS REVERSAL BY MAGNESIUM
AND CALCIUM IONS (52)

Concentration of anion, $M \times 10^3$	1.0 ml. C_1 dilution	Per cent lysis at final cation * concentration												
		No cation added	Mg^{2+}				Ca^{2+}							
			0.17	0.83	4.2	21	0.10	0.19	0.50	0.83	2.5	4.2	12.5	21
Citrate	None	43												
	0.9	34	97											
	0.9	7	86	92	7		65		16		4		1	
	9.0	0	1				20							
	9.0	0	0	1	83		1		1	2		1		
Pyrophosphate	None	41												
	0.2	10	96	92	93	6	19	15		6		0		
	2.0	0	0	44	92	11	1	2		1		7		

* Concentrations of magnesium and calcium ions, $M \times 10^3$; concentrations normally present were estimated at approximately $0.005 \times 10^{-3} M$ each.

It is now clear that certain biological fluids or tissue extracts which enhance lytic activity (20) do so by contributing magnesium ion (52). This is shown by the data in Table VIII. Conversely, the lytic system is also sensitive to procedures which reduce the concentration of magnesium ion. For example, anions like citrate (52), pyrophosphate (22,52), hexametaphosphate (22), and fluoride (83), which form insoluble or undissociated salts with magnesium ion, exert an anticomplementary effect, i.e., they inhibit lytic activity (Table

TABLE VIII

ENHANCEMENT OF COMPLEMENT ACTIVITY BY GUINEA PIG SERUM DIALYZATE OR BY CHICK EMBRYO ALLANTOIC FLUID WITH AND WITHOUT ADDITION OF OPTIMAL QUANTITIES OF CALCIUM AND MAGNESIUM IONS (52)*

Cation added, μg .	Serum dialyzate 1			Serum dialyzate 2			Allantoic fluid		
	None	1:10	1:35	None	1:10	1:25	None	1:10	1:25
None	125	181	128	104	148	122	117	203	174
Ca^{2+} , 50							116	194	168
Mg^{2+} , 100	245	255	254	190		230	226	237	232
Ca^{2+} , 50; Mg^{2+} , 100							238	236	243

* Values given are 50% units per milliliter.

VII). The mode of action of other anticomplementary substances, however, cannot as yet be explained. For example, the inhibiting effect of γ -globulin on C' (15) is of interest in view of the therapeutic use of this plasma fraction. A recent report by Waring and Weinstein (80), however, indicates that injection of γ -globulin does not depress the 50% titer of human C' .

While the function of magnesium ions in the lytic process is obscure, the necessity for the presence of magnesium ion to bring out lytic activity is one of several ways in which the lysis of red cells by C' and antibody resembles many enzymic reactions.

Hemolytic studies are usually carried out at 37–38°C. but recent work (52) has shown that slightly higher C' titers are obtained at 32–35°. The effect of temperature changes in the vicinity of 37° is fortunately not pronounced so that it is adequate even in accurate work to control the temperature of incubation to $\pm 0.2^\circ\text{C}$. As might be expected the speed of hemolysis decreases as the temperature diminishes and the lytic process is arrested at 0–5°C. This is, however, not the case for fish C' which lyses well even at 0°C. (14).

In addition to careful adjustment of the dose of erythrocytes, the amount of antibody for sensitization and control of the concentration of sodium chloride and magnesium ion in the system, one other condition must be considered, namely, the volume in which the lytic reaction is carried out. It is sometimes thought that a given amount of C' will lyse a certain dose of sensitized cells, but this is not so since the C' will lyse fewer erythrocytes if these are contained in a larger volume. For example, 250 million sensitized cells (50% of the total dose of 500 million) were lysed in a volume of 2 ml. by 0.00368 ml. guinea pig C', but in a volume of 3, 5, or 7.5 ml. this number of cells required 0.00473, 0.00675, or 0.00925 ml. C', respectively (52). This relation is described by a linear equation of the form:

$$y = 0.00170 + 0.0010x$$

in which y is the amount of C' required for lysing 250 million cells and x is the volume in which the reaction is carried out. While the mechanism of this phenomenon is not clear the explanation might be found in a shift of equilibrium between free and bound C' in the lytic system.

It is now evident that the lack of accuracy and reproducibility frequently encountered in investigations on C' can be overcome by adequate control of the conditions discussed in the preceding section. The only constituent of the lytic system which is not stable is C' itself. However, this difficulty has also been overcome, since guinea pig C' sealed in glass ampoules and stored with solid carbon dioxide, remains constant in titer for at least several weeks (52). The use of such a pretitrated stock supply of C' makes it possible to eliminate the usual daily titration with much saving of time and labor.

B. MECHANISM OF IMMUNE HEMOLYSIS

Investigators have often proposed an enzymic mechanism for immune hemolysis. A number of observations make such a mechanism plausible. In comparisons of lytic agents such as saponin or oleate with C' and antibody, it is found that 1.9×10^{10} molecules of saponin or 2.9×10^{10} molecules of sodium oleate are required for the lysis of a single red cell (24), while only about 25,000 molecules of C' and 500 of antibody (42) are needed. In enzymic reactions the ratio of enzyme to substrate is, of course, small. Hence the far smaller numbers of C' and hemolysin molecules than those of nonenzymic saponin

or sodium oleate required for hemolysis are in accord with the assumption of enzymic activity for either C' or hemolysin.

The necessity for magnesium ion as a cofactor of the hemolytic system also constitutes a point of similarity to many enzymic processes. This similarity is also shown in the kinetic studies mentioned above. These indicate that the velocity of immune hemolysis is directly proportional to the concentration of C'. In unsuccessful attempts to elucidate the possible enzymic nature of C' Yenson (83) found that certain enzyme inhibitors such as cyanide, monochloroacetate, and iodoacetate failed to inactivate C'. Although efforts to identify C' with any enzyme known to occur in blood serum have failed (56d), this should not discourage further enzymologic attack.

III. Participation of Complement in Union of Antigen and Antibody

Morse (55) was among the first to point out that the precise quantitative methods for the measurement of the hemolytic activity of C' pioneered by Leschly (47) and Brooks (7,8) could be utilized for quantitative estimations of C' fixation. Since that time there have been numerous publications on the subject but the techniques evolved have found only limited application, probably because of their complexity and the difficulties encountered in their interpretation. It would seem, therefore, desirable to review the principles involved, since a better understanding of C' fixation through application of quantitative methods should contribute to our knowledge of the functional role of C' as an aid to immunity.

In the usual fixation test two 100% units of C' are mixed with a specified amount of immune serum and antigen. After incubation, a standard dose of sensitized red cells is added and the mixture is incubated again. What happens now depends on how much, if any, C' has been fixed or destroyed during the first period of incubation. If less than one unit has been fixed, complete lysis occurs and the test is recorded as negative. If all of the C', i.e., two units, has been taken up by the immune system, no lysis takes place, and the test is positive. Fixation of an amount of C' intermediate between one and two units leaves enough only for partial lysis, and within these limits the test is susceptible of quantitative treatment. Thus, the test in its usual form is quantitative, within a limited range, and the only improvement necessary is an increase in range of operation so that

widely different amounts of immune system may be tested. Wadsworth, Maltaner, and Maltaner (79) and Rice (70) have accomplished this by adjusting the amount of C' to the strength of the immune system so that partial lysis is obtained in every case, permitting quantitative evaluation. Their method indicates how much C' must be incubated with antibody and antigen so that a specified degree of hemolysis (i.e., 50%) will occur in the lytic reaction. A test of this kind is, however, complicated and difficult to interpret in terms of the union between antibody, antigen, and C' because it involves simultaneous changes of two variables, namely, immune complex and C' . As noted by Morse (55) more C' is taken up by a given quantity of immune complex when more C' is present and, therefore, tests with varying amounts of C' are not directly comparable. This effect also causes difficulties in the calculation of results since, in the incubation of varying amounts of C' with immune system, the loss of hemolytic activity due to fixation is not directly proportional to the amount of C' in each tube. As a consequence, the different amounts of active C' remaining after incubation in the series of tubes are no longer related to one another in the proportions in which they were distributed in the tubes, resulting in an abnormal lytic curve and therefore yielding values of $1/n$ differing from 0.2. For this reason special conversion factors are necessary for each kind of test. In our laboratories a considerable simplification is attained by conducting the test with a constant number of units of C' (53). This, in effect, is a return to the usual fixation procedure, but, in order to obtain a wide range of operation, not 2, but 50 or even 100 units of C' are employed. However, unlike the usual test in which sensitized cells are added to the entire reaction mixture, the new method requires determination of the amount of C' remaining free after fixation by analysis of an aliquot portion chosen so as to yield a partial degree of lysis with the standard dose of sensitized cells.

The analytical determination of the hemolytic activity of complement remaining after fixation presents no difficulty, for it could be shown that the hemolytic curve of the residual free complement is described by equation 1 (page 89) with the normal value of 0.2 for the constant $1/n$ (53). Therefore, calculations are exactly the same as in an ordinary quantitative titration of C' (52). The test is carried out in two steps: first, 50 or 100 units of C' are incubated with antigen and antibody; second, an aliquot portion of the reaction mixture

is incubated with the standard dose of sensitized red cells. From the degree of lysis obtained, the number of residual free C' units is calculated. Finally, this value is subtracted from 50 or 100, the initial dose, to yield the number of C' units fixed.

Initial studies (53) given in Table IX, were conducted with a constant amount (0.25 μ g.) of pneumococcus type III polysaccharide (S III) and varying quantities of rabbit antibody. It was found that fixation followed a linear course in the range of 0.5 to 1.5 μ g. of antibody nitrogen and within these limits quantitative measurements of these minute amounts of antibody could be made. In the region of extreme antigen excess no fixation of C' occurred at these very low antibody levels. Studies made with a constant known amount of rabbit antibody against pneumococcus type III and varying quantities of S III have shown that the fixation of C' varies with the amount of antigen added to the constant quantity of antiserum. The reaction passes through a region of maximum fixation. Excess antigen causes inhibition of fixation, just as it does in specific precipitation. Similar studies by the method of Wadsworth *et al.* have been published by Rice (70). The zone of optimum fixation, however, is no more sharply delimited than is the equivalence zone in the precipitin reaction (32a,b). The method of Rice (71), which involves determination of the amount of antigen for optimum fixation, can therefore yield only an approximate estimate of the potency of an immune serum.

Studies (53,72) also indicate that the capacity of a given weight of antibody to fix C' varies in different sera and that the antibody present in an individual serum can be fractionated into portions of different C'-fixing potency (74). It would, therefore, seem invalid to utilize antibody nitrogen values for testing empirical equations of C' fixation such as those used by Rice and Sickles (73,74). Analyses show that the amount of C' fixed by a given quantity of immune system increases as the initial dose used in the reaction is made larger. As already stated, this effect was noted by Morse (55), who considered that the proportion of C' fixed, and not the absolute amount, gives a true measure of the quantity of immune system. However, the present work shows that the proportion of C' fixed to that remaining free diminishes as the initial dose of C' is increased, although there is an increase in the absolute quantity fixed. It thus appears that the amount of immune complex and the quantity of C' fixed are

TABLE IX

UNITS OF GUINEA PIG COMPLEMENT FIXED BY 0.25 MICROGRAM S III AND VARYING QUANTITIES OF RABBIT ANTIPNEUMOCOCCUS TYPE III SERUM C-28 (53)

Controls	Titration of residual C'		Veronal buffer added, ml.	1.0 ml. hemolytic system added; incubation 60 min.* at 37°C.				Hemolysis, %	Factor†	Residual C', units	C' fixed, units	C' fixed, corr. to 50 units
	Dilution of reaction mixture	Volume of dilution tested, ml.										
Serum (4 µg. antibody N) S III (0.25 µg.) Buffer	1 + 9	2.0	4.5	1.0 ml. hemolytic system added; incubation 60 min.* at 37°C.				45.9	0.970	48.5		
		2.5	4.0					71.8	1.202	48.1		
	1 + 9	2.0	4.5					49.5	0.998	49.9		
	1 + 9	2.5	4.0					72.8	1.215	48.6		
Antibody N used, µg.		2.0	4.5	1.0 ml. hemolytic system added; incubation 60 min.* at 37°C.				47.1	0.978	48.9		
		2.5	4.0					71.8	1.202	48.1		
								Mean: 48.7				
0.71	1 + 9	2.0	4.5	1.0 ml. hemolytic system added; incubation 60 min.* at 37°C.				39.5	0.915	45.8	3.5	3.6
0.94	1 + 9	2.5	4.0					63.3	1.113	44.5		
1.18	1 + 9	3.0	3.5					52.4	1.02	40.8	7.8	8.0
1.66	1 + 9	3.0	3.5					73.9	1.23	41.0		
2.36	1 + 9	3.5	3.0	1.0 ml. hemolytic system added; incubation 60 min.* at 37°C.				60.7	1.090	36.3	12.8	13.1
	1 + 9	3.5	3.0					74.9	1.241	35.5		
	1 + 9	3.5	3.0					43.5	0.950	27.1	21.9	22.5
	1 + 9	4.0	2.5					56.9	1.056	26.4		
3.78	1 + 4	3.0	3.5	1.0 ml. hemolytic system added; incubation 60 min.* at 37°C.				59.5	1.078	18.0	30.7	31.5
	1 + 4	3.5	3.0					76.0	1.260	18.0		
	1 + 4	3.5	3.0					47.5	0.982	14.0	34.7	35.6
	1 + 4	4.0	2.5					64.4	1.125	14.0		

* In other experiments 45 minutes was shown to be adequate.

† See references 52 and 53.

related according to their relative proportions just as are antigen and antibody in the precipitin reaction. This is not surprising in view of the multivalence of the immunologic reagents (32a,b) perhaps including C' itself.

IV. Purification of Complement and Isolation of Its Components

The question of the multiple nature of complement and the characterization and separation of the components have been active subjects for investigation ever since Ferrata (19) in 1907 dialyzed C' against water and found that the resulting precipitate and solution were inactive separately but caused hemolysis of sensitized red cells when recombined. These two components, now designated C'1 and C'2 in agreement with Pillemer and Ecker (62), rather than "mid-piece" and "endpiece" (56), remain the only components of C' which have been recognized and characterized by their actual separation as well as by their activities in mediating hemolysis.

The development of modern methods for the separation of the protein constituents of the serum complex has been a long and tedious process. Accordingly, many years elapsed between the first separation of C'1 and C'2 and the actual isolation of one of these components in a state of relative purity (63). The euglobulin fraction of guinea pig serum insoluble at a concentration of 1.39 *M* ammonium sulfate was further purified through removal of lipides by centrifugation in the cold and dialysis against phosphate buffer at ionic strength 0.02 and pH 5.2. The insoluble portion contained the C'1 activity and was redissolved and reprecipitated at a concentration of 1.22 *M* ammonium sulfate, redissolved in 0.5% sodium chloride, and again dialyzed against the same buffer at pH 5.2. It was then homogeneous in the ultracentrifuge. 98% of the protein present was contained in a single component on electrophoresis, so that the preparation appeared to be pure C'1 by these physicochemical criteria. The properties of the fraction are given in Table X. The yield was 400 mg. from 1070 ml. serum.

The serum proteins soluble in 2.0 *M* ammonium sulfate but insoluble at a concentration of 2.2 *M* contained most of the C'2 and C'4 activities of the serum. Dialysis of the material against conductivity water yielded a greenish gel containing the activities of C'2 and C'4 but these could not be separated. After several reprecipitations the

preparation showed 98% in a single component in the Tiselius electrophoretic apparatus, but did not appear homogeneous in the ultracentrifuge. It is therefore uncertain whether this unusual euglobulin of high carbohydrate content and high levorotation contains C'2 and C'4 as integral portions of the same molecules or whether further separation might be possible. The C'4 function could, however, be separated by its greater resistance to heat than that of C'2.

C'3 was distributed among the various fractions isolated.

TABLE X
PROPERTIES OF PURIFIED COMPONENTS OF GUINEA PIG COMPLEMENT (63)

Properties	C'1	C'2, 4
Type of globulin	eu-	muc-eu-
(NH ₄) ₂ SO ₄ , per cent saturation, 0°	37	58
Mobility, * $\times 10^6$	-2.9	-4.2
Sedimentation, S	6.4	
[α] _D , degrees	-29	-193
Carbohydrate, %	2.7	10.3
Fraction of total serum protein, %	0.6	0.2
Temperature at which destroyed, 30 min.	50°	50° (C'2) 66° (C'4)

* PO₄³⁻, pH 7.7, μ = 0.2.

The quantities of C'1 and C'2, 4 isolated by Pillemer *et al.* (63) totalled 0.8% of the guinea pig serum proteins. While these amounts probably do not represent 100% yields of material of 100% purity, a combination rarely achieved in biochemical preparations, they agree remarkably well with the analytically estimated average percentages (28) of C' in guinea pig serum, namely, 0.4 to 0.7%. A complex present in so small a proportion of the serum proteins could not be expected to show in electrophoretic or ultracentrifugal diagrams of whole guinea pig serum.

A peculiarity of isolated C'1 was its inactivity in concentrated solution and full potency at high dilutions (63). This property is possibly related to the anticomplementary nature of euglobulins in general and may account for the analytical observations of Heidelberg (28), namely the larger the volume of guinea pig serum present, the smaller was the quantity of C' nitrogen per milliliter fixed by specific precipitates (see pages 75-76).

The Pillemer-Ecker group has also undertaken the fractionation of human C', but up to the present no component has been isolated in a satisfactory state of purity (66). In the large-scale fractionation of

human plasma undertaken at Harvard C'1 is said to be concentrated in fraction III-2 with thrombin and C'2 in fraction IV with hypertensinogen (13).

V. Titration of the Components of Complement

Principles for the titration of the components of C' were clearly enunciated by Hegedüs and Greiner (25). The reasonable hypothesis was propounded that the titer of a C' is limited by the component present in lowest titer, and that each of the four components might be titrated independently by addition of the other three components in excess, so as to make the component in question the one present in lowest titer. Required reagents were prepared by standard methods from guinea pig C'. However, doubts arose as to the adequacy of technique used for measuring the various components, and the conclusions reached as to the relative quantities of the components in guinea pig C' were not confirmed (4). It was, therefore, evident that existing methods for the titration of the components of C' required revision. Accordingly estimations were made of the titers of the components present in "midpiece," "endpiece," and in the so-called "specifically inactivated" complements (65) prepared by different methods, since this was a prerequisite to an understanding of these reagents and to their use in quantities adequate to provide, in optimal excess, the components desired. Moreover, the greatest care was taken to use each reagent well below its anticomplementary range. Emphasis on this rudimentary precaution was indicated by the lack of any positive evidence that it was observed in numerous recent investigations.

Details of the methods used are fully given (4). A reagent for the titration of a given component should not contain the component to be titrated, but should contain an excess (*i.e.*, more than one unit per dose employed) of each of the remaining components. Four reagents are required (see 25): reagent 1, containing C'2,3,4, for the titration of C'1; reagent 2, containing C'1,3,4, for the titration of C'2; reagent 3 with C'1,2,4, for the titration of C'3; and reagent 4, containing C'1,2,3, for the titration of C'4. A reagent should produce no lysis of sensitized red cells when used alone in several times the quantity employed in actual titrations. Reagent 1 was prepared by combination of E,* which furnished C'2 and some C'3 and C'4,

* E = "endpiece," M = "midpiece," the soluble and insoluble portions, respectively, of dialyzed or diluted C'.

with heated serum which supplies extra C'3 and C'4. Reagent 2 consisted of M* plus heated serum. In each instance the heated serum was added separately to the tubes in which the tests were carried out. "Zymosan"-treated (16,61) C' was employed as reagent 3, while ammonia- or hydrazine-treated C' served as reagent 4.

For purposes of control, the adequacy of each reagent should be established by experiment, a condition not met in some studies (16,25,29). This requires testing each reagent (R) with every other reagent, as follows: (a) R1 + R2; (b) R1 + R3; (c) R1 + R4; (d) R2 + R3; (e) R2 + R4; (f) R3 + R4. If complete hemolysis of added sensitized red cells follows, the first test demonstrates the presence of one or more units of C'2 in the amount of R1 used, and one or more units of C'1 in R2. Similarly, the second test establishes the presence, in adequate amount, of C'3 in R1 and C'1 in R3. If all tests result in complete hemolysis, it is certain that each of the four reagents actually contains the three components which it is designed to supply.

Below the anticomplementary limit, the quantity of a reagent should be chosen in a range in which the titer of the component remains independent of the amount of reagent employed. The compositions of average preparations of each reagent are given below. For example, E, which is used in R1, showed the following average composition:

Source and method of preparation	C'2 titer, units/ml.	C'3 titer, units/ml.	C'4 titer units/ml.
Human, dialysis	110	35	2400
dilution	115	45	2400
Guinea pig, dialysis	140	60	3500
dilution	220	60	2100

It is evident that E is generally deficient in C'3, since less than 100 units are present per milliliter and 0.1 ml. of a 1:10 dilution (the amount generally employed) contains less than one unit of C'3. The addition of heated complement, by supplying C'3, serves to remedy the deficiency and is especially necessary with human E. Guinea pig E + heated guinea pig serum yielded the best R1, since guinea pig E contains more C'2 than human E, and heated guinea pig serum is not anticomplementary and supplies more C'3 than heated human serum. Guinea pig C' inactivated for twenty minutes at

56°C. usually titered 100 to 300 units of C'3 and about 500 to 2000 units of C'4. The C'4 titer of heated human serum was similar, but the C'3 content was very low.

The average composition of M follows:

Source and method of preparation	C'1 titer, units/ml.	C'3 titer, units/ml.	C'4 titer, units/ml.
Human, dialysis	1000	90	350
dilution	1000	40	130
Guinea pig, dialysis	350	120	100
dilution		100	

Again it is seen that M should be combined with heated C' for use as R2, owing to its deficiency of C'3 and often of C'4 as well. This applies especially to human M, which was preferred for R1 since it is not anticomplementary. Guinea pig M, when freshly prepared, should not be anticomplementary, but often becomes so if kept in solution, or if made alkaline:

AVERAGE COMPOSITION OF ZYMOSAN-TREATED COMPLEMENT

Source	C'1, titer, units/ml.	C'2, titer, units/ml.	C'4, titer, units/ml.
Human	2000	100	2000
Guinea pig	1000	260	2000

If at least 0.02 ml. (0.1 ml. of 1:5 dilution) is used, this reagent functions as R3.

AVERAGE COMPOSITION OF AMMONIA-TREATED COMPLEMENT

Source	C'1, titer, units/ml.	C'2 titer, units/ml.	C'3 titer, units/ml.
Human	2000		
Guinea pig	1200	300	100

The anticomplementary effect of guinea pig M, when encountered, was found to be somewhat greater toward homologous C' than human C'. Addition of heated guinea pig serum greatly reduced this anticomplementary action of guinea pig, human, and sheep M, the only ones tested. Human M is seldom anticomplementary toward human C' but may be toward guinea pig C'. Human and guinea pig C'

, treated with zymosan were not anticomplementary nor was ammonia-treated guinea pig serum. Human serum treated with ammonia was always anticomplementary toward guinea pig serum, but not against human serum, guinea pig E, or heated guinea pig serum. With the limits established within which the various reagents might be used, titration of the four components was now possible.

a. First Component, C'1. Complement dilutions most suitable for titration of C'1 were found to be between 1:200 and 1:500. Increasing amounts were set up with an amount of reagent 1, usually between 0.2 to 0.5 ml., previously found to contain an optimal excess of C'2, 3, and 4. Reagent 1, prepared by use of a volume of guinea pig E such as given above, and 0.05 ml. of 1:5 heated guinea pig serum, was found preferable to that from human E. As prepared and used with 0.2 ml. of suspension containing 125 million sensitized sheep erythrocytes, guinea pig R1 usually contained about 3 units of C'2, 3 of C'3, and 50 to 100 of C'4. The C'1 titrations carried out on zymosan- and ammonia- or hydrazine-treated sera showed that these inactivation procedures for C'3 and C'4, respectively, are not entirely specific since all remaining components were reduced.

b. Second Component, C'2. R2, for the titration of C'2, was preferably composed of human M, in amounts of 0.025 to 0.1 ml. of a 1:5 dilution, plus 0.05 ml. of 1:5 heated guinea pig serum. As so prepared, the amounts used contained 5 to 20 units of C'1, 2 to 3 of C'3, and 5 to 10 of C'4. R2 prepared with nonanticomplementary lots of guinea pig M may also be employed. Titration of C'2 in whole C' may be carried out with heated guinea pig serum alone, since C' contains excess C'1. However, for titration of C'2 in E, the complete reagent, R2, is necessary. Sheep M plus heated guinea pig serum is also useful as R2.

c. Third Component, C'3. R3, optimal for this titration, was prepared by treatment of guinea pig C' with zymosan (16) according to (61), although the corresponding reagent from human C' was also used in some instances. 0.05 ml. of 1:5 dilution (based on the guinea pig C' used) appeared optimal for the titration of C'3 in human C' and 0.1 ml. of the same dilution for the titration in guinea pig C'. The former quantity of reagent contained roughly 10 units of C'1, 2 to 3 of C'2, and 20 of C'4.

d. Fourth Component, C'4. R4 consisted preferably of am-

monia-treated guinea pig C' since the corresponding reagent from human C' was anticomplementary (see 16) and contained too little C'2 and 3. Usually 0.2 ml. R4 at 1:10 dilution was used, the component content being 20 to 30 units of C'1, 6 of C'2, and 2 of C'3. Although C'4 is considered a heat-stable component, the effect of heating sera at 56°C. for different periods is appreciable:

Human C'			Guinea pig C'		
Period heated, min.	C'4 titer, units/ml.	Activity, %	Period heated, min.	C'4 titer, units/ml.	Activity, %
0	3300	100	0	3300	100
5*	3000	90	5*	3000	90
10	1500	45	10	2000	60
15	800	25	15	1300	40
20	600	20	20	500	15
30	130	4	30	130	4

* Two minutes was added to each heating period for the sample to come to 56°C. after immersion in the bath.

Some of the more complete sets of titration values on individual complements are given for comparison in Table XI. Average titers found for human and guinea pig sera are given in Table XII.

TABLE XI

TITERS OF HUMAN AND GUINEA PIG COMPLEMENTS AND THEIR COMPONENTS (4)

Source and number	C', units/ml.	C'1, units/ml.	C'2, units/ml.	C'3, units/ml.	C'4, units/ml.
Individual, human	70	2700	80	>130	3,000
" " "	100	5000	200	350	2,500
" " 5	80	5000	130	350	2,500
" " 7	100	4000	100	250	3,000
Pool, human 4	100	2000	130	>200	2,700
Pool, guinea pig	300		800	350	6,000
" " "	500		500	600	17,000
" " " 7	270	1000	270	300	7,000
" " " 8	300	1300	400	300	4,000
" " " 9	400	1600	400	400	10,000

In the course of the study (4) each of the reagents used was subjected to scrutiny for its actual titer of the component or components it was expected to furnish, and its anticomplementary properties were checked in order to ascertain whether or not it might safely be used in concentrations high enough to supply the needed amounts.

The conditions in this way become analogous to those preferentially employed in the estimation of other biologically active substances. For example, comparisons of enzyme concentrations should be made at relatively high substrate concentrations so that the apparent enzyme values obtained are independent of substrate concentration (6). Similarly in studies of blood clotting, components other than the one to be measured should be present in excess so that the clotting time may vary only as a function of the amount of unknown component in the system.

TABLE XII
AVERAGE TITERS OF COMPLEMENT AND COMPONENTS (4)

Source	Whole C', units/ml.	C'1, units/ml.	C'2, units/ml.	C'3, units/ml.	C'4, units/ml.
Guinea pig	350	2300*	450	370	6000
Human	100	3700*	170	250	4000

* Average titer with R1 (guinea pig E plus heated guinea pig serum), the optimal reagent.

The four reagents used (25) for the study of the complements of a large number of animal species were prepared from guinea pig C' by standard methods: E for the titration of C'1, M for the titration of C'2, guinea pig serum from which C'3 had been removed by the action of snake venom for the titration of C'3, and ammonia-treated guinea pig serum, lacking in C'4, for the titration of C'4. No definite evidence was given, however, that the amounts of reagents used actually contained an excess of the desired components, or that they were always used below their anticomplementary concentrations.

It was found that E, the standard reagent for C'1, was often too deficient in C'3 to provide more than a limiting titer, so that this remained the component of lowest titer in the mixture and was actually measured instead of C'1. It is believed that this accounts for the finding (25) that the titer of C'1 in guinea pig C' is lower than that of C'2, whereas the actual titer of C'1 is now found to be very high. It was also noted that M, the standard reagent for C'2, often failed to yield a measure of this component since it furnished too little C'3, in nonanticomplementary quantities, to raise the titer of this component in the test mixture above that of C'2. M was often deficient in its content of C'4, as well.

These defects proved to be readily remediable by addition of

guinea pig serum which had been heated at 56°C. for twenty minutes, which sufficed to render negative all hemolytic tests for C'1 and C'2. This not only provided an excess of C'3 (and C'4 if not already present), but also greatly reduced the often appreciable anticomplementary activity of M prepared from guinea pig serum. Since human M was not anticomplementary and usually showed a considerably higher titer of C'1 than did guinea pig M, the reagent (R2) preferably used in these studies for the estimation of C'2 consisted of human M + heated guinea pig serum. The human E obtained as a by-product in the preparation of M is difficult to use owing to its low titer of C'2, so that for the titration of C'1, guinea pig E + heated guinea pig serum is recommended as the reagent (R1).

It was also found that in the so-called "specific inactivation" (65) of C'3 by "zymosan" and of C'4 by ammonia or hydrazine the other components of C' suffered more or less reduction as well. It is therefore proposed to abandon the inaccurate designation "specifically inactivated" complements, and call these reagents R3 and R4, respectively, numbering them as in R1 and R2, according to the components measured with their aid.

Although it is a simple matter to test a treated serum or reagent for anticomplementary action by its depression of the titer of whole C', the evaluation of anticomplementary activity is not without complication. A treated serum or reagent may exert an enhancing action or an anticomplementary one, or it may have no effect at all on the titer of C'. Enhancing effects are attributable to the addition of the component, or components, present in lowest titer, *i.e.*, the titer-limiting component. Both enhancing and anticomplementary effects could, however, exist side by side in the same solution, but, of course, only the net effect could be demonstrated. In such an event the reagent might be anticomplementary toward guinea pig C', for example, in which C'3 is usually the limiting component, but enhancing toward human C', in which C'2 is the component in lowest titer. This would also explain the effect of heated guinea pig C' in diminishing the anticomplementary action of the guinea pig M, since C'3 is added and this is the limiting component in guinea pig C'. Possibly these circumstances also afford an explanation for the increase in titer of the C' by heated guinea pig serum. While this would be expected to increase the titer of guinea pig C', in which C'3 appears on the average to be the component of lowest titer (Tables

XI and XII) although the titer of C'2 is not much higher, the reason for the increase is not so apparent in the case of human C', in which C'2 is the limiting component. However, the titer of C'3 is not very much greater. Since it is stated that C'3 is not fixed when C' is taken up in hemolysis (64,81), it is possible that a considerable excess of this component is essential for full hemolytic activity, perhaps by driving back dissociation of a loose combination. Heated guinea pig serum would provide such an excess.

Other explanations are, however, possible, and the effect of heated serum requires further study.

In the course of this investigation (4) the mutual equivalence of each of the components of human and guinea pig C' for the hemolysis of sensitized sheep red cells was also demonstrated. It is not, however, always as convenient to use a human component as a guinea pig component.

Ecker and Pillemer and their co-workers have independently arrived at acceptance of underlying principles similar to those of Bier *et al.* (4) (private communication, Dr. E. E. Ecker; see also 17), so that necessary

revisions of their published conclusions as to the components of C' may be awaited.

Throughout this section emphasis has been laid on the titers of C' and its components. Since volume units have been used, and not units of weight, as, for example, in the quantitative method for whole C' (3,28,37,39), the data are purely relative and give no clue to the actual content or concentration of any component. Nor do the titers even yield information as to the relative concentrations of the components, since equal quantities of each component are not necessarily required for hemolysis. The treatment given does, however, in spite of its shortcomings and inaccuracies, place the estimation of the components of C' on a rational basis.

Figure 4 shows average titers of human and guinea pig C' and ap-

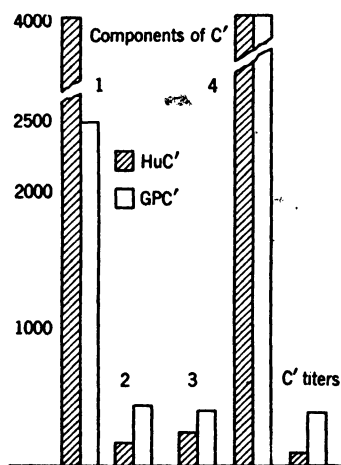


Fig. 4. Titers of human (HuC') and guinea pig (GPC') complements and their components (56c).

proximate titers of their components as determined according to Bier *et al.* (4). It is evident that the titer of human C' is limited by C'2 and that of guinea pig C' by C'3. The highest titers are those of C'1 and C'4, and in guinea pig C' the titer of the latter is often far higher than that of C'1, which furnishes much of the nitrogen added to specific precipitates by C'. In spite of this, only one third as much C'2, 4 as of C'1 was isolated from guinea pig C' in the experiments of Pillemer *et al.* (p. 102). If the yields were representative of actual quantities in guinea pig serum this could only mean that far less C'4 than C'1 is required for hemolysis. The titers, then, vary inversely as the amounts needed to lyse red cells and are, therefore, wholly illusory as to the relative or absolute quantities involved. Conceivably, if any component of C' acts as an enzyme it is C'4, which is present in high titer and performs its function in minute amounts.

VI. Relation of Complement to Bactericidal and Opsonic Effects of Fresh Sera

Recent attempts have been made to analyze the factors responsible for the opsonic (18,49) and bactericidal (18) activities of fresh sera and so to place these determinations on a more quantitative basis. The conclusion was reached (49) that the same C' is the active factor as in hemolysis, that calcium ion is essential, and that C'4 is likewise needed, contrary to earlier belief (23). It was also concluded (18) that C'4 is required for opsonization but that sera lacking C'3 show little or no loss of opsonic activity.

VII. Conclusion

It is shown in the present review that application to the study of C' of rigorous microanalytical methods yielding the result in weight units has led to an estimate of the actual quantity of C' in the sera of various species and that the data not only furnish an insight into the mechanism of immune hemolysis but, applied to C' fixation, are also compatible with quantitative theory. Precise spectrophotometric methods of measurement of the unit of C' causing 50% hemolysis have led to a clear demonstration of the activating effect of magnesium ions and other bivalent ions and the consequent implication of an enzymic hypothesis for the mechanism of hemolysis. New light has also been thrown, by use of these methods, on the fixation of C' by antigen-antibody combinations. Also, a more careful analysis of

the factors involved in the estimation of the components of C' has placed these determinations on a rational and useful basis, although the methods are still crude and fail to conform to the criteria of quantitative analysis. Isolation of relatively pure C'1 and a fraction containing C'2,4 have added to knowledge of the nature, amounts, and properties of these components of C'. It would seem that many of the tools are now available for the resolution of some, at least, of the remaining perplexities posed by the strange and baffling complex known as complement.

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DEHYDROPEPTIDASES

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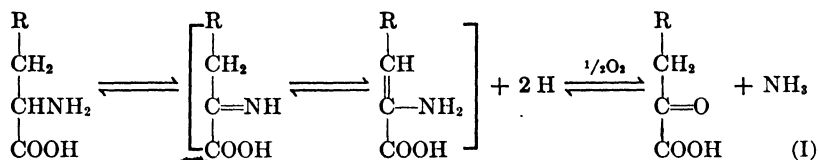
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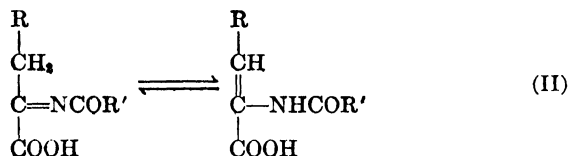
I. Introduction

The physiological equilibrium between α -amino acids on the one hand, and of α -keto acids and ammonia on the other, first demonstrated by Neubauer (46) and by Knoop (41), was assumed by the latter to involve the intermediate formation of an α -imino acid and two hydrogen atoms. For all amino acids except glycine, the possibility that this imino acid might itself be in tautomeric equilibrium

with the corresponding α,β -unsaturated amino acid (α -aminoacrylic acid and its higher homologs) was simultaneously suggested by Dakin (17) and by Bergmann (9)—see reaction (I), where R represents any one of the natural side chains on the β carbon of the amino acids, i.e., C_6H_5- , $(CH_3)_2CH-$, $SH-$, etc.



The tautomers represented in the brackets in Reaction I are extremely unstable and have never been isolated. They may be presumed to hydrolyze spontaneously into the corresponding α -keto acid and ammonia. When, however, the nitrogen atom is acylated, the resulting compounds are entirely stable in aqueous solution under ordinary conditions. Such unsaturated compounds which are represented as follows:

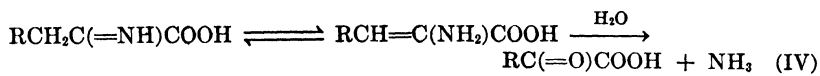
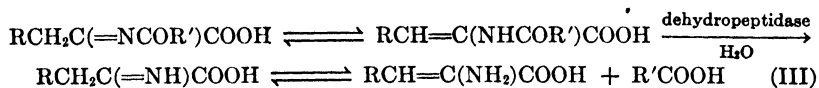


where R' may be an aliphatic or aromatic radical, all contain a substituted amide bond, and because of this fact, plus that of their unsaturated character, have been given the designation of *dehydropeptides* (3).

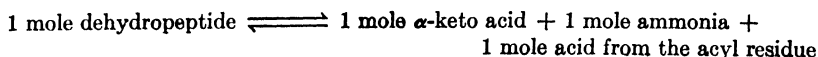
On boiling dilute mineral acid solutions of the dehydropeptides, the corresponding α -keto acid, the corresponding acid from the acyl residue, and ammonia are formed. The same products are formed from dehydropeptides with suitable R and R' substituents when they are incubated under mild physiologic conditions with aqueous preparations of a wide variety of animal or plant tissues. Under such conditions, certain dehydropeptides were found to be enzymically hydrolyzed at the peptide bond with great rapidity in preparations of every animal and plant tissue studied (51). To the enzyme responsible, the designation of *dehydropeptidase* has been given (6).

The dehydropeptidases act specifically upon peptides containing an α,β -double bond adjacent to the substituted amide bond, and are

distinct from those peptidases, such as dipeptidase, carboxypeptidase, etc., which catalyze the hydrolysis of the peptide bond uniting the normally saturated α -amino acids. The hydrolysis of the dehydropeptides may be considered to occur in two consecutive steps, the first (III) being enzymic, and the second (IV) being spontaneous:



or:

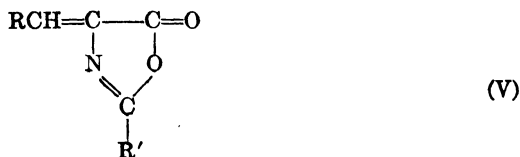


Before describing the enzymic hydrolysis of the dehydropeptides, it might be useful to review briefly the organic-chemical background of this class of compounds, and to indicate the relation of such compounds to analogous and derived products of physiologic interest, *i.e.*, diacylaminopropionic acids, α -keto acids, and pyruvoylpeptides.

II. Preparation and Properties of Dehydropeptides and Related Compounds

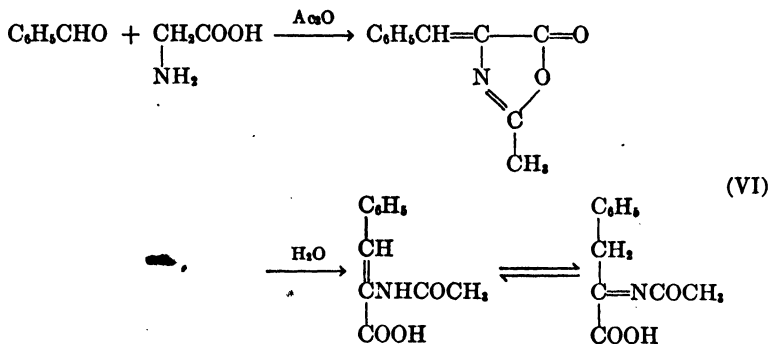
A. SYNTHESSES INVOLVING AZLACTONE FORMATION

The formation of the first unsaturated azlactone was reported in 1883 by Plöchl (49), who condensed benzaldehyde with hippuric acid in the presence of acetic anhydride (V). The structure of the



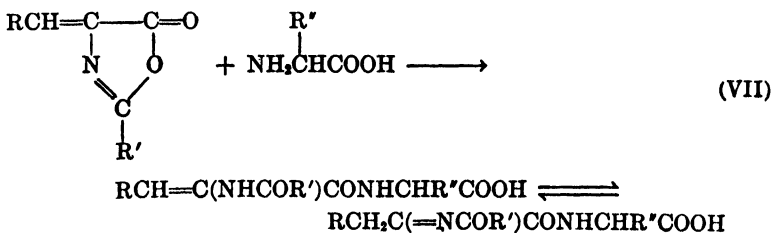
product and the designation *azlactone* given it was due to Erlenmeyer (19), who extended the method of preparation to other aldehydes, and who established the usefulness of the azlactones as intermediates in the synthesis of α -keto and α -amino acids (21), compare (16). Erlenmeyer and Früstück (20) noted that the azlactone ring in many instances could be opened in the presence of cold alkali, leading to the formation of the corresponding α,β -unsaturated *N*-acylated acid,

and by this means were the first to prepare a dehydropeptide, in this instance, *N*-acetyl- α -aminocinnamic acid (VI).



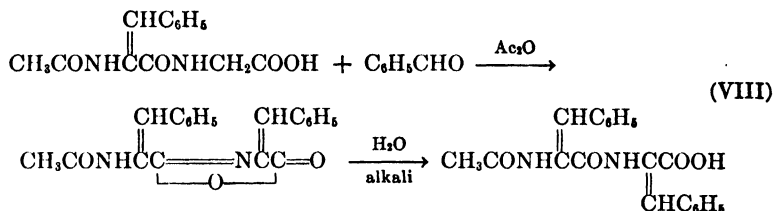
The study of the azlactones, and their usefulness in preparing dehydropeptides of various kinds, was undertaken by Bergmann and co-workers, and described in a notable series of publications. This work may be considered as comprising three parts: (a) extension of the dehydropeptide chain at the carboxyl end by permitting the azlactone ring to open in an aqueous solution of the sodium salt of an amino acid or in a nonaqueous solution of an amino acid ester (4,8,10); (b) extension of the dehydropeptide chain at the carboxyl end by treating the dehydropeptide with an aromatic aldehyde in the presence of acetic anhydride and sodium acetate, thereby obtaining the azlactone of a doubly unsaturated dehydropeptide, which on alkaline hydrolysis yields the free peptide with two dehydrogenated amino acid residues (18); (c) dispensing with the need for employing an aldehyde in the azlactone synthesis by employing either an *N*-halogenated-acyl peptide, an *N*-acylated peptide of serine, or an *N*-halogenated-acyl peptide of phenylserine (5,9,18):

(a) The unsaturated azlactones apparently react rapidly with primary amines, in much the same fashion as do acid anhydrides, per-



mitting the formation of dehydropeptide chains lengthened at the carboxyl group of the original dehydropeptide (VII). The added amino acid may also be coupled to the dehydropeptide in the form of its ester, the coupled-product ester yielding the free peptide after saponification.

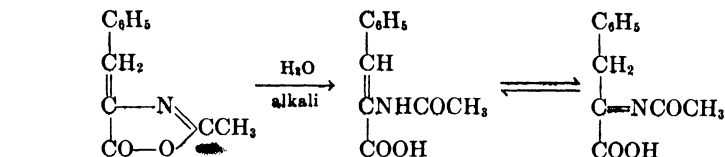
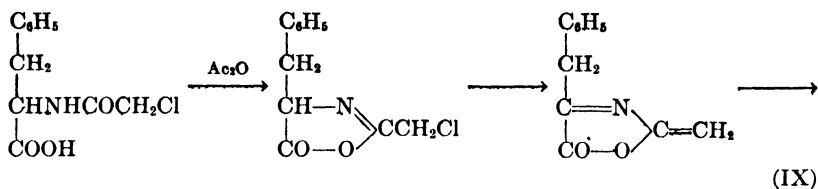
(b) Dehydropeptides with a glycine residue at the carboxyl end of the peptide chain, when treated with an aromatic aldehyde at 40°C. in the presence of acetic anhydride yield the azlactones of unsaturated peptides containing more than one double bond. Thus, *N*-acetyl- α -aminocinnamoylglycine plus benzaldehyde yields, by way of the intermediate azlactone, *N*-acetyl- α -aminocinnamoyl- α -aminocinnamic acid (acetyldehydrophenylalanyldehydrophenylalanine) (VIII).



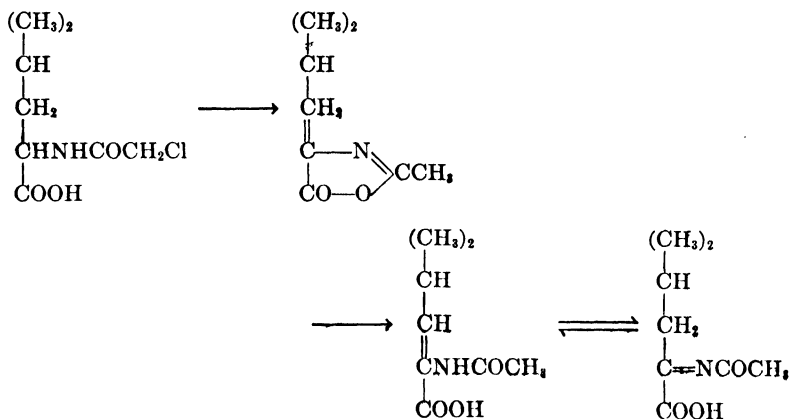
The peptides and azlactones containing one or several double bonds may be expected to exist in several stereoisomeric forms. The use of *p*-hydroxybenzaldehyde in place of benzaldehyde makes accessible peptides containing the dehydrogenated tyrosine residue. Peptides containing a dehydrogenated aliphatic amino acid can also be prepared by utilizing the azlactone derived from the corresponding *N*-halogenated acylamino acid, a reaction described in the following section.

(c) By using *N*-halogenated acylamino acids with acetic anhydride, the use of the aldehydes in the preparation of the unsaturated azlactones can be avoided. Thus *N*-chloroacetylphenylalanine or *N*-chloroacetylleucine when warmed with acetic anhydride readily form the azlactone by splitting off not only water but hydrogen chloride (IX). In the presence of pyridine, the formation of the azlactone from the *N*-halogenacylated amino acids proceeds very rapidly (16).

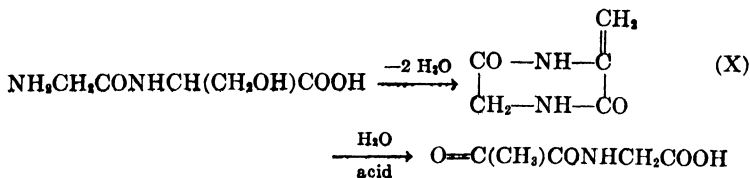
Another way of dispensing with the use of the aromatic aldehydes is to employ *N*-acylated peptides of serine. The presence of the hydroxyl group on the β carbon of serine provides a means whereby



or

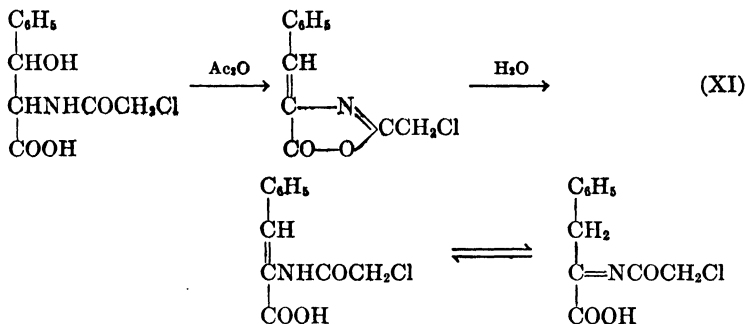


through removal of the elements of water on adjacent carbons an α,β double bond can be introduced. Thus, glycylserine is readily cyclized (5) by loss of two molecules of water to form the 3-methylene-2,5-diketopiperazine, which is in effect a cyclic anhydride of α -aminoacrylic acid with glycine. On mild hydrolysis with mineral

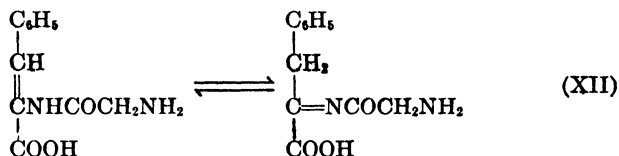


acid, the diketopiperazine ring is opened and pyruvoylglycine is formed (X).

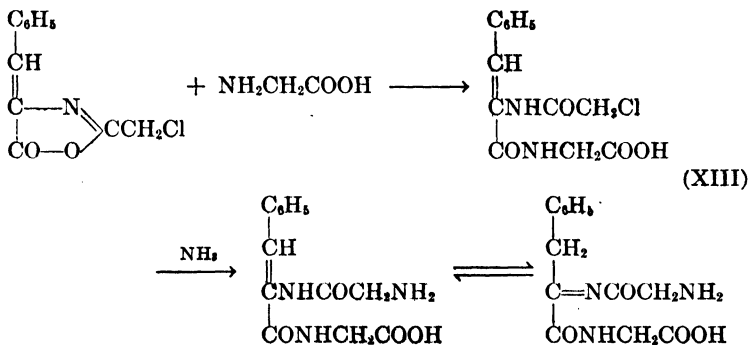
Phenylserine can serve in place of serine in this connection. When *N*-chloroacetylphenylserine is dehydrated under carefully controlled conditions by acetic anhydride, the corresponding azlactone is formed with the chloroacetyl residue intact. On hydrolysis with dilute alkali, the ring is opened, and an α -halogenated acyldehydropeptide



(*N*-chloroacetyldehydrophenylalanine) is formed (XI). On amination with aqueous ammonia, the corresponding glycyldehydrophenylalanine is produced (XII).



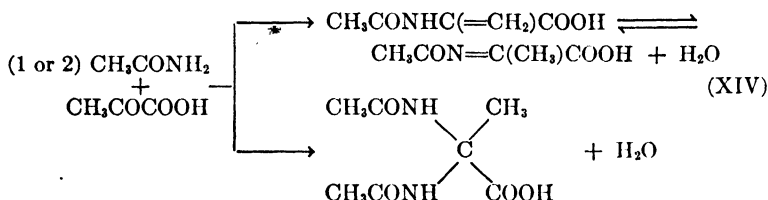
Glycyldehydrophenylalanine was the first representative of this class of substances which possessed both a free α -amino group and a free α -carboxyl group, and thus facilitated and encouraged the use of such substances in studies of tissue metabolism. Peptides in which



the dehydrophenylalanyl residue was in the middle of the chain could be prepared by letting the azlactone ring in XI open in the presence of sodium glycinate (see *a*, above) obtaining thereby *N*-chloroacetyldehydrophenylalanylglycine, which on subsequent amination yielded glycyldéhydrophenylalanylglycine (XIII).

B. SYNTHESSES INVOLVING CONDENSATION OF AMIDES AND α -KETO ACIDS

Bergmann and Grafe (3) noted that heating a mixture of acetamide and pyruvic acid under reduced pressure led to the formation of a mixture of α,α -diacetaminopropionic acid and α -acetaminoacrylic acid (XIV). Depending therefore on whether one or two molecules

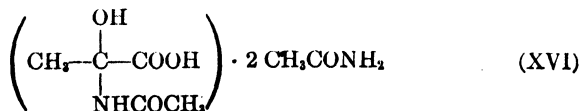


of amide reacted per molecule of pyruvic acid, acetaminoacrylic acid or the diacetaminopropionic acid, respectively, was formed. The two products could be separated from each other by fractional crystallization in ethyl acetate. From the pure diacetaminopropionic acid the dehydropeptide can be prepared by warming with glacial acetic acid (XV). The reversibility of reaction (XV) was demonstrated

$$(\text{CH}_3\text{CONH})_2\text{C}(\text{CH}_3)\text{COOH} \rightleftharpoons \text{CH}_3\text{CONHC}(=\text{CH}_2)\text{COOH} + \text{CH}_3\text{CONH}_2 \quad \text{(XV)}$$

by Shemin and Herbst, who prepared diacetaminopropionic acid by heating a mixture of acetaminoacrylic acid and acetamide (56).

In a careful study of the interaction of acetamide with pyruvic acid, Herbst (39) showed that the first step in the condensation probably consists of a complex of one molecule of α -hydroxy- α -acetaminopropionic acid with two molecules of acetamide (XVI). The complex on heating *in vacuo* decomposes to the products noted.

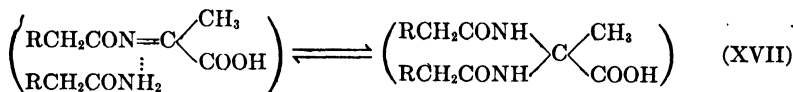


That other acid amides would condense with pyruvic acid by simply warming mixtures of the two reactants was shown by Bergmann and Grafe (3), who obtained a mixture of chloroacetaminoacrylic acid and α,α -dichloroacetaminopropionic acid from chloroacetamide and pyruvic acid, by Nicolet (47), who obtained a mixture of α,α -dibenzoylaminoacrylic acid and α -benzoylaminoacrylic acid from benzamide and pyruvic acid, and by Martell and Herbst (44), who prepared a number of benzyl carbonyl derivatives by employing benzyl carbamate with a variety of ketones. Condensation of pyruvic acid with formamide leads to the formation of α -hydroxy- α -formaminopropionic acid, which decomposes to yield α -formaminopropionic acid (56a).

According to Martell and Herbst (44), the interaction between amide and keto acid involves the primary addition of amide to the carbonyl group, followed either (a) by direct replacement of the hydroxyl group by another amide residue (see XVI) to form the diacylaminoacrylic acid, or (b) by elimination of water with formation of unsaturated intermediates (acylated aminoacrylic acids), to which, with proper conditions, a second mole of amide may add. Shemin and Herbst had noted the reversibility of equation XV (56). They were, however, unable to demonstrate this reversibility when the keto acid was phenylpyruvic acid, for acetamide would not condense with acetaminocinnamic acid. This discrepancy was ascribed (44) to the tendency of the phenyl group in the aminocinnamic acid derivatives to hold the side chain double bond in a position conjugated with the aromatic ring, and thus to prevent the tautomeric shift of the double bond to the carbon-nitrogen position. Thus, although diacylaminoacrylic acids may be prepared by heating two molecules of amide with one of phenylpyruvic acid (44), and acetaminocinnamic acid can be prepared by heating one molecule of acetamide with one of phenylpyruvic acid (56), it is apparently impossible to prepare the diacylaminoacrylic acids by condensing acylaminocinnamic acids with amides. For this class of compounds, alternative *a* above appears to hold, whereas it seems probable that alternative *b* may hold for pyruvic acid derivatives.

The preparation of acylaminoacrylic acids (dehydropeptides) from the corresponding diacylaminoacrylic acids suggests that one of the amide residues in the diacylaminoacrylic acids is relatively labile. In other words, it might appear that the diacylaminoacrylic

acids are probably acylaminoacrylic acids to which an amide residue is more or less loosely attached at the double bond (XVII).



However, under ordinary circumstances in aqueous solution, the diacylaminoacetic acids are quite stable compounds. When one of the acyl groups is removed and the amino group is free, as in α -

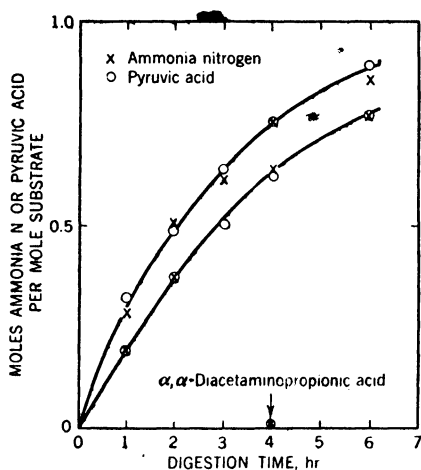
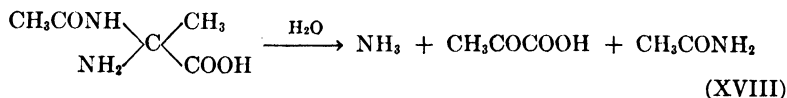


Fig. 1. Spontaneous hydrolysis of α -acetamino- α -aminopropionic acid to equivalent amounts of ammonia and pyruvic acid (32) at 37°C.: upper curve, with rat kidney extract; lower curve, in water.

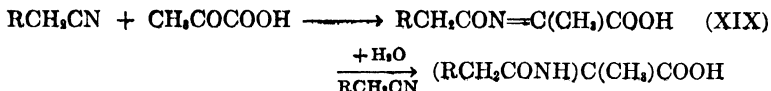
acetamino- α -aminopropionic acid (2) the compound is very unstable in aqueous solution, and as shown by Gonçalves and Greenstein (32) spontaneously decomposes into one mole of ammonia, one mole of pyruvic acid, and one mole of acetamide (Fig. 1). In the presence of aqueous rat kidney extract, this spontaneous decomposition is accelerated (XVIII).



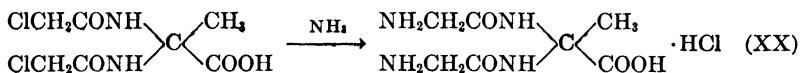
In order to prepare large quantities of pure diacylaminoacetic acids unmixed with the corresponding acylaminoacrylic acids, Gonçalves and Greenstein (32) employed a reaction first described by Böttlinger in 1881 (13) which consisted in adding an excess of nitrile

to a solution of pyruvic acid in chilled concentrated sulfuric acid. On pouring the reaction mixture into ice, the desired diacylamino-propionic acid appears in good yield. In this way, considerable amounts of crystalline diacetaminopropionic acid (34) and dichloroacetaminopropionic acid (32) were prepared.

The Bötttinger synthesis may be considered as proceeding by two steps, the first a condensation of nitrile with keto acid to form the corresponding dehydropeptide, followed by the fixation of a second molecule of nitrile in the presence of water (which forms the nascent amide) on the double bond of the dehydropeptide, yielding finally the diacylamino-propionic acid (XIX). On treatment with aqueous



ammonia (XX), the α,α -dichloroacetaminopropionic acid yielded α,α -diglycylaminopropionic acid hydrochloride, which was stable and neutral in reaction in aqueous solution and which possessed the following dissociation constants at 25°C. (32): $pK_1 = 1.8$, $pK_2 = 8.1$, $pK_3 = 8.1$. The constant pK_1 refers to the dissociation of the carboxyl group, and the constants pK_2 and pK_3 refer to the dissociation

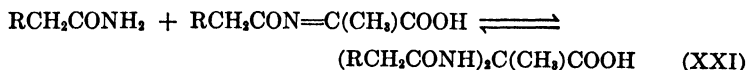


of the two amino groups. The fact that $pK_2 = pK_3$ suggests that the amino groups ionize independently of each other.

Analogous reactions involving DL-chloropropionitrile were studied by Price, Errera, and Greenstein (53), and led to the preparation of α,α -di(DL-chloropropionylamino)propionic acid, which on amination formed successively α -(DL-chloropropionylamino)- α -(DL-alanyl-amino)propionic acid and α,α -di(DL-alanyl-amino)propionic acid hydrochloride.

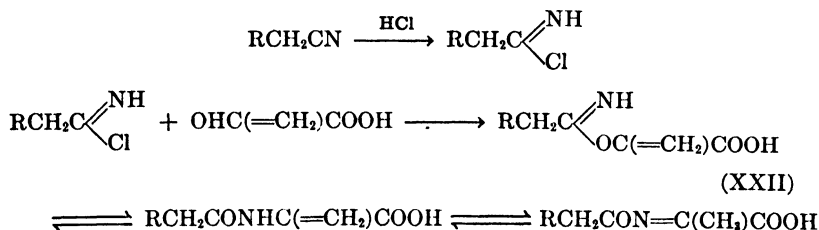
Some attention has been paid to the diacylamino-propionic acids because of their relation to the acylaminoacrylic acids (dehydropeptides). As will be described below, diglycylaminopropionic acid is a substrate for powerful enzymic activity in animal tissues, and the mechanism of the breakdown of this substrate may well involve the intermediate formation of the dehydropeptide, glycyl- α -aminoacrylic acid (glycyldehydroalanine). The reversible relation between the

dehydropeptides and the corresponding diacylamino propionic acids may therefore be generally expressed as in reaction (XXI).



C. SYNTHESSES INVOLVING CONDENSATION OF NITRILES AND α -KETO ACIDS

A relatively simple method of preparing certain dehydropeptides in high yield and purity was developed by Price and Greenstein (51). This was based on mixing the desired nitrile with an excess of pyruvic acid and saturating the mixture with dry hydrogen chloride gas. In a brief period of time the dehydropeptide is nearly quantitatively formed. The reaction may be interpreted as due to the primary formation of the imino chloride of the amide, which then reacts with the enolic form of the pyruvic acid (XXII).



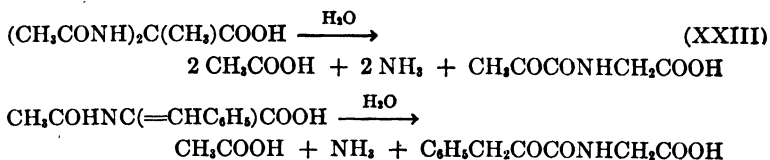
In this fashion considerable quantities of chloroacetyl aminoacrylic acid (chloroacetyldehydroalanine) and α -chloropropionyl aminoacrylic acid have been prepared, from which the corresponding glycyldehydroalanine and DL-alanyldehydroalanine have been derived by amination in aqueous ammonia (51). So far as can be observed, the reaction is limited to the use of nitriles with a halogen substituent on the α carbon, for acetonitrile failed to react under these conditions with pyruvic acid. For the preparation of acetaminoacrylic acid (acetyldehydroalanine) therefore, it is best to first prepare the diacetaminopropionic acid and to subsequently split this compound into the dehydropeptide and acetamide by means of warm glacial acetic acid (3,34). For the preparation of the α -halogenated dehydropeptides, the reaction of Price and Greenstein is simplest and most direct.

The reactions given in XXII are essentially similar to those which yield the diacylamino propionic acids by the Böttlinger synthesis

(XIX). In the former, the pyruvic acid is in excess and moisture is carefully excluded. In the latter the nitrile is in excess, and the presence of water is necessary to add the second amide residue to the dehydropeptide.

D. PYRUVOYLPEPTIDES

Dipeptides of α -aminoacrylic acid on complete hydrolysis by acids or by enzymic action yield, among other products, ammonia and pyruvic acid (III, IV, page 119). Tripeptides in which the dehydroalanyl residue is in the middle of the peptide chain would, on controlled hydrolysis only of the acyl residue, yield pyruvoylpeptides. Thus, Bergmann and Grafe (4) noted that α, α' -diacetaminopropionylglycine on warming with hydrochloric acid yielded acetic acid, ammonia, and pyruvoyl glycine, while similarly Fruton and Bergmann (28) noted that α -acetaminocinnamoylglycine yielded acetic acid, ammonia, and phenylpyruvoylglycine.

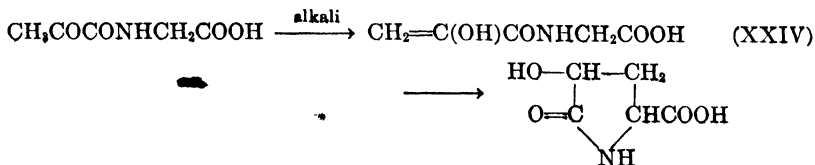


Fruton and Bergmann (28) showed that such α -keto acid peptides as phenylpyruvoyl-L-phenylalanine are very susceptible substrates for crystalline carboxypeptidase. On reductive amination, Herbst noted that the pyruvoylpeptides are converted to the corresponding saturated peptides, pyruvoylglycine thus yielding DL-alanylglycine (40).

Another procedure whereby keto acid peptides are obtained is the mild hydrolysis of the cyclic anhydride of aminoacrylic acid with glycine or alanine (5) (see reaction X).

Errera and Greenstein (24) observed that pyruvoylglycine possessed a $pK = 3.3$ at 25°C ., readily formed a crystalline dinitrophenylhydrazone, was easily hydrolyzed quantitatively by hot hydrochloric acid to pyruvic acid and glycine, and in aqueous solution at $pH < 10$ yielded a characteristic ultraviolet absorption spectrum with maxima at 2400 \AA and at 3400 \AA . When the aqueous solution was brought to a $pH > 10$, the compound no longer yielded a dinitrophenylhydrazone, on boiling with hydrochloric acid no pyruvic acid or glycine could be recovered, and the absorption maxima in the

ultraviolet disappeared. The pK at 25° was still 3.3, and the nitrogen content of the isolated material was the same as that of pyruvoylglycine. The original properties of the pyruvoylglycine which were lost in alkaline solution were not restored on acidification. These irreversible phenomena were interpreted as being due to an intramolecular rearrangement of the α -keto acid peptide which occurs in alkali, to form the γ -hydroxypyrrolidone carboxylic acid (XXIV).



The position of the hydroxyl group in the pyrrolidone ring is similar to that in hydroxyproline. It is possible that the selective hydrolysis of peptides containing an α,β -dehydrogenated amino acid residue would, under physiologic conditions, lead to the production of keto acid peptides, which under suitable circumstances could be converted to saturated peptides or to amino acids with ring compounds of the pyrrole type. That certain peptides such as chloroacetyl glycine may readily be converted *in vitro* to pyrrole compounds had been shown by Bergmann, Zervas, and Lebrecht (12), and these authors stressed the ease of interconversion of the various types of protein-building materials.

Subsequent studies by Errera and Greenstein (25) on the absorption spectra in the ultraviolet of aqueous solutions of phenylpyruvic acid and of phenylpyruvoylglycine showed that, at pH 7.8, the former possesses an absorption band at 2850 \AA , and the latter at 2500 \AA . When these solutions were brought to pH 11.5, the spectra altered immediately, and a well-marked maximum at 3250 \AA appeared for both compounds. On bringing the solutions back to pH 7.0, the original spectra for both compounds was restored. The alkalization of pyruvic acid, of phenylpyruvic acid, and of phenylpyruvoylglycine is reversible; that of pyruvoylglycine is irreversible.

E. ULTRAVIOLET ABSORPTION SPECTRA OF THE DEHYDROPEPTIDES

As described above (II, III, page 119), the dehydropeptides exist in aqueous solution in a tautomeric equilibrium of several forms. It

would therefore be expected that their absorption spectra in the ultraviolet would reveal characteristic properties and indeed this was found to be the case. Carter and Greenstein (14) noted that the aliphatic dehydropeptides possessed a characteristic absorption in the ultraviolet with a maximum at 2400 Å (Fig. 2). The shape of the absorption curve for the acylated peptides of α -aminoacrylic acid

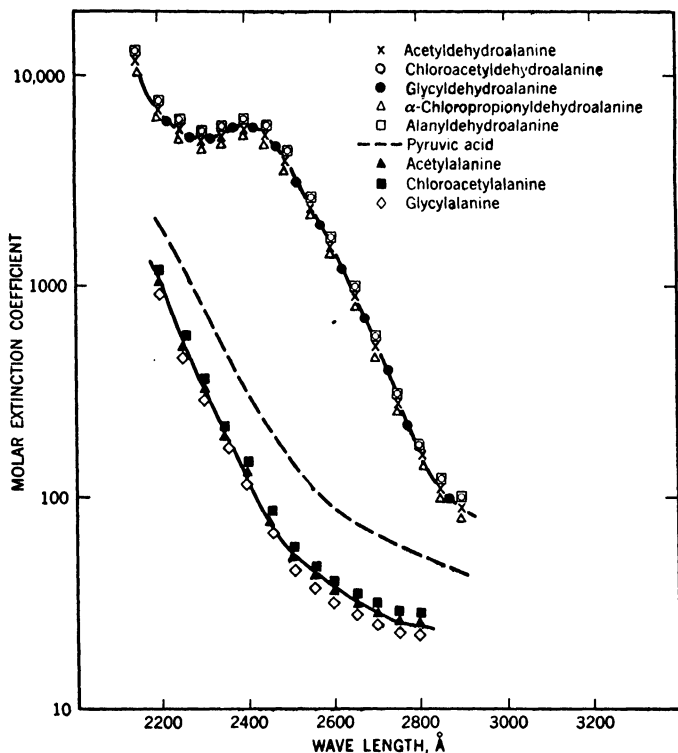


Fig. 2. Absorption curves in the ultraviolet of peptides of dehydroalanine and of alanine at pH 7.0 (51).

was practically independent of the nature of the acyl residue (50). The saturated analogs of the dehydropeptides reveal little absorption, and that not characteristic, in the ultraviolet region.

The shape of the absorption curves for acylated peptides of α -aminophenylacrylic acid is considerably different from that of the aliphatic dehydropeptides (Fig. 3). The curves for the aromatic

dehydropeptides possess a minimum at 2400 Å and a maximum at 2750 Å. In general, these curves resemble those of the saturated analogs of the phenylated amino acids (Fig. 3), but the amount of absorption of the dehydropeptides is considerably greater, and the

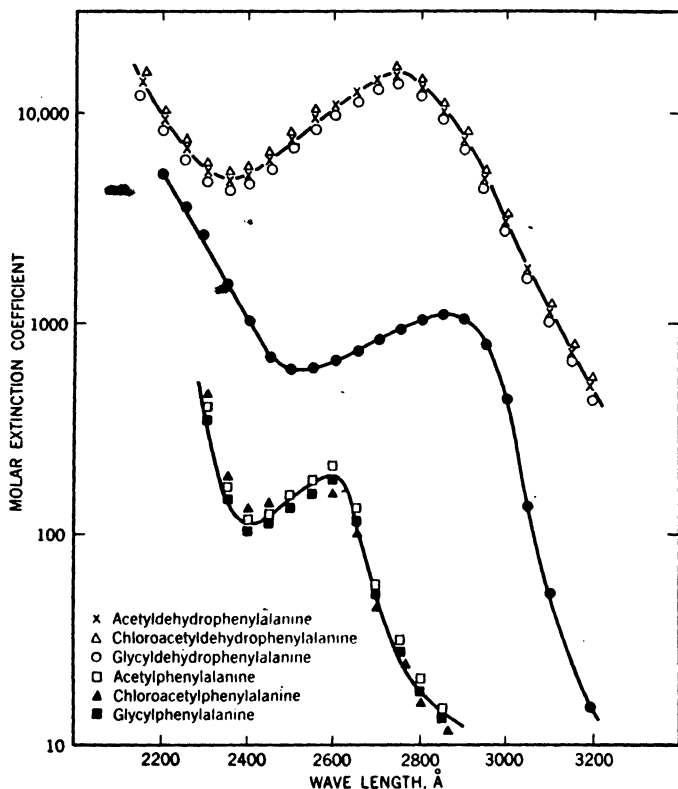


Fig. 3. Absorption curves in the ultraviolet of peptides of dehydrophenylalanine and phenylalanine at pH 7.0 (51). Middle curve is for phenylpyruvic acid.

maximum is shifted from 2600 to 2750 Å. The presence of the resonating double bond adjacent to the phenyl group enormously enhances the absorption of ultraviolet light by the entire molecular system. As in the case of the aliphatic dehydropeptides, the shape of the aromatic dehydropeptides is largely independent of the nature of the acyl residue (51).

The absorption curves in the ultraviolet for acetyldehydrotyrosine

were determined at 2 pH values by Fruton *et al.* (29) (Fig. 4).

In contrast with characteristic absorption of acetyldehydroalanine, that of acetyldehydroaminobutyric acid, acetyldehydrovaline, and acetyldehydroleucine is only general, although in considerable excess over that of their saturated peptide analogs (29,52a).

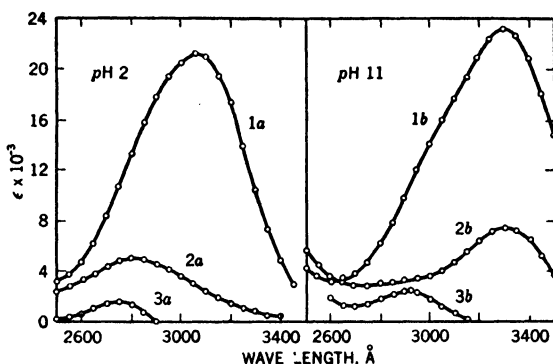


Fig. 4. Absorption curves in the ultraviolet of acetyldehydrotyrosine and related compounds in acid (pH 2) and alkaline (pH 11) solution. Curves 1a and 1b, acetyldehydrotyrosine; curves 2a and 2b, *p*-hydroxyphenylpyruvic acid; curves 3a and 3b, acetyltyrosine (29).

F. CHEMICAL REACTIVITY OF DEHYDROPEPTIDES

As Bergmann and collaborators have shown (3,5,9,10), the dehydropeptides readily take up catalytic hydrogen at the double bond to form the saturated DL-peptide analogs. Alkaline permanganate and elemental bromine are also taken up at the double bond.

Quantitative studies by Eiger in the writer's laboratory on the amounts of Br consumed by acetyldehydroalanine revealed that nearly the theoretical amount of Br was taken up by this compound. No splitting of the halogenated compound appeared to occur (production of ammonia), and the characteristic absorption of the original dehydropeptide in the ultraviolet disappeared, a more general type of absorption taking its place.

Chloroacetyldehydroalanine treated with aqueous ammonia at 0° or at 25°C. invariably yields the corresponding glycyldehydroalanine. However, chloroacetyldehydroalanine, treated with aqueous methylamine, yields at 0° the corresponding sarcosyldehydroalanine,

and at 25° yields sarcosyl- α -amino- β -methylaminopropionic acid hydrochloride (52). When treated with aqueous dimethylamine at either 0° or 25°, chloroacetyldehydroalanine yields *N,N*-dimethylglycyl- α -amino- β -dimethylaminopropionic acid hydrochloride (52). Thus, methylamine and dimethylamine may, under relatively mild conditions, substitute not only at the α -carbon but also at the double bond of chloroacetyldehydroalanine, producing saturated peptides.

Addition of mercaptan at the double bond of dehydropeptides has been reported by Nicolet (48). A synthesis of cystine was achieved by heating acetyldehydroalanine with benzylmercaptan.

III. Enzymic Hydrolysis of Dehydropeptides and Related Compounds

A. DISCOVERY AND SPECIFICITY OF DEHYDROPEPTIDASE ACTIVITY

In 1930, Bergmann, Schmitt, and Miekeley (8), with the statement that "es scheinen uns biologische Umbau- und Abbaureaktionen von Peptiden möglich, die nicht die völlige Hydrolyse bis zu den einfachen Aminosäuren voraussetzen," investigated the effect of incubating commercial pancreatin with a number of peptides of dehydrophenylalanine (aminocinnamic acid). There appeared to be a very slow but definite splitting of glycyldehydrophenylalanine into glycine, ammonia, and phenylpyruvic acid. Chloroacetyldehydrophenylalanine was apparently not split at all under these conditions.

The study of the enzymic hydrolysis of glycyldehydrophenylalanine was carried further by Bergmann and Schleich (6), who noted the following: (a) purified and highly active preparations of dipeptidase, aminopolypeptidase, carboxypeptidase, trypsin, and pepsin, which rapidly split peptides of the normally saturated amino acids, had no effect upon glycyldehydrophenylalanine, (b) extracts of animal kidneys were a good source for the enzyme or enzymes which attacked glycyldehydrophenylalanine, while extracts of yeast, intestinal mucosa, liver, stomach, muscle, brain, and fresh pancreas were largely inactive to this substrate, (c) the kidney preparation is rapidly inactivated by cyanide, (d) tripeptides, such as glycyldehydrophenylalanylglycine, in which the dehydrogenated amino acid is in the middle of the chain, are not enzymically attacked, thereby suggesting that the action of the effective enzyme may require the presence of a free carboxyl group, (e) the pH optimum for the splitting of glycyl-

dehydrophenylalanine in kidney extract is about 7.5, and (f) neither acetyldehydrophenylalanine nor chloroacetyldehydrophenylalanine were split by kidney extracts.

The possibility that the substrate was split not at the peptide bond but rather at the C-N bond on the cinnamic acid chain to yield glycine amide and phenylpyruvic acid was dismissed by Bergmann and Schleich (7) on the ground that glycine amide with the enzyme preparations used yielded ammonia too slowly to satisfactorily account for the ammonia noted in digests with glycyldéhydrophenylalanine.

On the basis of these findings, Bergmann and Schleich announced in 1932 (6) the discovery of an enzyme in kidney which would specifically split glycyldéhydrophenylalanine into ammonia, phenylpyruvic acid, and glycine, and to which the designation *dehydropeptidase* was given.

B. DISTRIBUTION AND ACTIVITY OF DEHYDROPEPTIDASES

From 1932 to 1944 no further attention was paid to the topic of the enzymic hydrolysis of the dehydropeptides until this subject was taken up in the writer's laboratory. Greenstein and Leuthardt had noted (37) that the rate at which peptides of cystine incubated with fresh homogenates of rat liver yielded hydrogen sulfide, ammonia, and pyruvic acid was very close to that at which cystine itself yielded these products. Two possible explanations were: (a) the peptides were split to free cystine and the other amino acids at an extremely high rate by dipeptidase, carboxypeptidase, or aminopolypeptidase in the liver homogenates, and the cystine then metabolized, or (b) the cystine peptides were first desulfurated, yielding the corresponding dehydropeptides, which then by the action of powerful dehydropeptidase activity in the digest produced ammonia and pyruvic acid. The decision between these alternatives rested largely on the relative susceptibility of the saturated peptides and of the unsaturated peptides to enzymic hydrolysis in fresh tissue homogenates. When it was noted that the usual substrates for dipeptidase (DL-alanylglycine), carboxypeptidase (chloroacetyl-DL-tyrosine), and aminopolypeptidase (triglycine) were split relatively slowly, whereas glycyldéhydroalanine was split very rapidly, it appeared as if alternative b above might be correct (37).

With these observations, which in the case at least of the cystine peptides appeared to support the original Bergmann thesis that amino

acids might be metabolized while they are yet in peptide linkage, it was considered of interest to examine the susceptibility of a number of the dehydropeptides to the action of fresh homogenates from a wide variety of animal and plant tissues, utilizing quantitative methods of determination for following the splitting of the substrates and for observations on the end products of the reactions. It was observed that for one of these substrates at least, alanyldehydroalanine,

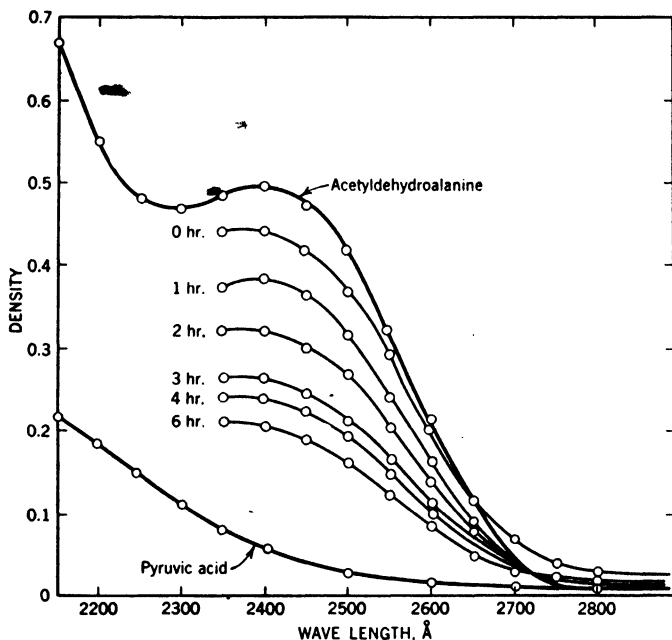


Fig. 5. Progressive change in the absorption curve of acetyldehydroalanine as a result of digestion with rat kidney extracts (34).

an enzyme existed in very high activity in every tissue studied, and that therefore dehydropeptidase activity was one of the most intensive as well as extensive intracellular metabolic systems in animal and plant tissues (14,51).

Dehydropeptidase activity in tissue extracts and homogenates can be measured equally well in either of two ways, chemically, by measuring the rate at which ammonia or keto acid appears in the digests (37,51), or spectrophotometrically, by measuring the rate of dis-

appearance of the absorption band at 2500 Å in the case of the aliphatic dehydropeptides (14,34,51) and at 2750 Å in the case of the peptides of dehydrophenylalanine (51). Figures 5 and 6 demonstrate the changes in the absorption curves of acetyldehydroalanine and of glycyldehydrophenylalanine as these substrates are progressively

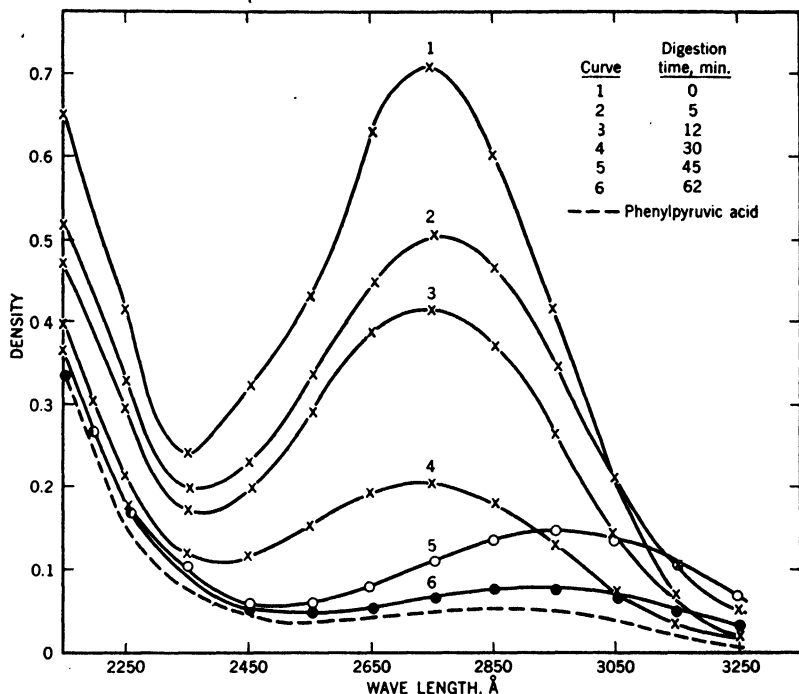


Fig. 6. Progressive change in the absorption curve of glycyldehydrophenylalanine as a result of digestion with rat kidney extracts (51).

split in tissue digests to products which include, respectively, pyruvic acid and phenylpyruvic acid. The shapes of the absorption curves in each case approaches, at the end of hydrolysis of the dehydropeptides, those of the respective keto acids.

A similar procedure was employed by Fruton, Simmonds, and Smith (29), who measured the dehydropeptidase activity of growing *Escherichia coli* cultures by noting the decrease in the absorption at two wavelengths of acetyldehydrotyrosine, namely, at 3050 Å and 2750 Å (see Fig. 4).

A note of caution must be suggested in dealing with the spectrophotometric determination of dehydropeptidase activity when glycyldehydrophenylalanine is used as substrate. Examination of

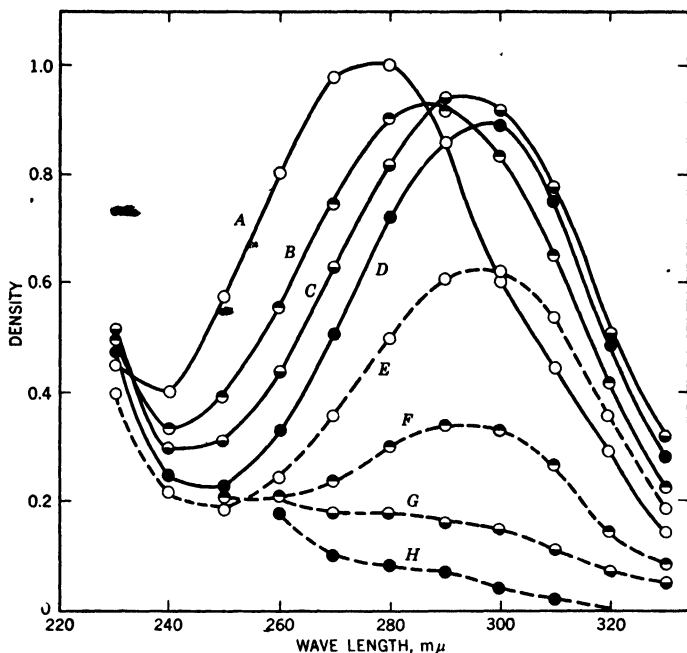


Fig. 7. Changes in absorption of glycyldehydrophenylalanine ($5.7 \times 10^{-5} M$) when incubated with purified dehydropeptidase I (0.00125 mg. enzyme N per cc. reaction mixture) at 38° in 0.02 *M* borate buffer pH 8.1 (55). Times in minutes: A = 0, B = 10, C = 20, D = 40, E = 100, F = 205, G = 720, H = 1140. Step A-C, enzymic (hydrolysis of peptide bond, no appearance of ammonia, inhibited by cyanide, heat-labile), compare reaction III; steps C-H nonenzymic (ammonia production, increased by heat, slowed by cold), compare reaction IV. Ordinate = observed optical density, 1-cm. cell.

Figure 6, curves 5 and 6, suggests that, during the course of the enzymic digestion of glycyldehydrophenylalanine, an absorption maximum at the longer wavelength of 2950 \AA appears. This phenomenon is more vividly illustrated by the data of Shack (55) obtained with a highly purified dehydropeptidase preparation and low substrate

concentration (Fig. 7). It would appear that the hydrolysis of this substrate may occur in two steps, each at a different rate, of which one step at least is enzymic, and that misleading kinetic data would occur if the hydrolysis is followed at only a single wavelength. The phenomena shown in Figures 6 and 7 may assist in explaining some of the anomalies noted by Yudkin and Fruton in their studies on the enzymic hydrolysis of glycyldehydrophenylalanine (58). It is possible that the stabilizing influence of the benzene ring adjacent to the double bond permits of the existence of one or more tautomeric forms, which in aliphatic compounds of this type would be much more unstable. Figure 5, for example, reveals no presence of any intermediate form during the digestion of acetyldehydroalanine. If the intermediate compound suggested in Figures 6 and 7 is actually the α -imino form of phenylalanine, reaction (I), due essentially to Knoop, would be experimentally realized.

The wide variety of tissues which possess dehydropeptidase activity, and the relative susceptibility of various dehydropeptides, is illustrated in Table I. The rate at which ammonia nitrogen appears in digests of the various tissues, over the region where the evolution of ammonia occurs nearly linearly with time, was employed to measure the dehydropeptidase activity. DL-Alanyldydroalanine, only one of whose optical components is split (51), and glycyldehydroalanine are hydrolyzed in extracts of all tissues studied (Table I). Glycyldehydrophenylalanine is hydrolyzed at a markedly slower rate. Chloroacetyldehydroalanine and DL- α -chloropropionyldehydroalanine are hydrolyzed only in extracts of kidney, liver, pancreas, and some plants, while acetyldehydroalanine is hydrolyzed in extracts only of kidney, liver, and mushrooms at a rate lower than that of chloroacetyldehydroalanine. Acetyldehydrophenylalanine and chloroacetyldehydrophenylalanine are apparently not affected in extracts of the tissues studied.

Fruton, Simmonds, and Smith (29) have shown that growing (but not resting) cultures of *E. coli* can metabolize acetyldehydrotyrosine and acetyldehydroalanine, but not acetyldehydrophenylalanine. The metabolic product of acetyldehydrotyrosine appeared to be a derivative of *N*-acetyltyramine in which the side chain had undergone oxidation.

Because of the relative distribution in tissues of the capacity to split glycyldehydroalanine, on the one hand, and chloroacetyldehydro-

TABLE I
SUSCEPTIBILITY OF VARIOUS DEHYDROPEPTIDES IN RAT AND PLANT TISSUE EXTRACTS (38a,51,52)*

Dehydropeptide	Micromoles ($\times 10$) substrate split/hour/mg. total N/ml. extract in									
	Kidney ¹	Liver	Pan- creas	Brain	Spleen	Muscle	Mush- rooms	Yeast ²	Beans ³	Sweet peas (seeds)
DL-Alanyldehydroalanine ⁴	1350	52	722	220	412	58	750	35	360	401
Glycyldehydroalanine	1620	60	530	72	331	38	150	12	42	72
Glycyldehydrophenylalanine	520	12	132	0	23	0	8	0	0	0
DL- α -Chloropropionyldehydroalanine ⁴	29	8	3	0	0	0	13	1	0	3
Chloroacetyldehydroalanine	100	28	10	0	0	0	40	4	2	7
Chloroacetyldehydrophenylalanine	0	0	0	0	0	0	0	0	0	0
Acetyldehydroalanine ⁵	18	3	0	0	0	0	6	0	0	0
Acetyldehydrophenylalanine	0	0	0	0	0	0	0	0	0	0
Sarcosyldehydroalanine	800	25	280	30	166	16	—	—	—	—
Chloroacetylglucyldehydroalanine	1580	50	450	40	240	28	—	—	—	—
Glycylglucyldehydroalanine	1660	60	500	64	320	35	—	—	—	—
Chloroacetyl-sarcosyldehydroalanine	0	0	0	0	0	0	—	—	—	—
Chloroacetylglucyldehydrophenylalanine	0	0	0	0	0	0	—	—	—	—
Chloroacetyl-DL-alanyldehydroalanine	85	26	43	18	54	43	—	—	—	—

* Digests consisted of 1 ml. aqueous tissue extract + 2 ml. 0.15 M borate buffer at pH 8.1 + 1 ml. of either water or 25 micromoles of substrate (50 micromoles of racemic substrates). Hydrolysis measured in terms of ammonia nitrogen above controls. Neither glycine nor glycylglycine yields ammonia under these conditions. Solutions of acetyl- and chloroacetylpeptides neutralized with sodium hydroxide before use. DL-Alanine yields no ammonia under conditions used with DL-alanyldehydroalanine since extracts were very dilute and periods of incubation very short.

¹ Identical digests with the various substrates under anaerobic conditions yielded almost identical results.

² Fleischmann brand.

³ Kentucky Wonder.

⁴ Rate calculated on basis of one optical component, since only one form is split (51).

⁵ Acetyldehydroaminobutyric acid, acetyldehydrovaline, and acetyldehydroleucine are not hydrolyzed (52a).

alanine, on the other, it is believed that there exist at least two dehydropeptidases (37). One of these, designated by Greenstein and Leuthardt (37) dehydropeptidase I, is present in all tissues, and for which as substrate glycyldehydroalanine is suitable; the other, designated dehydropeptidase II, is present in only a few tissues, and for this enzyme chloroacetyldehydroalanine is suitable as substrate. Confirmation of the presence of at least two separate dehydropeptidases with the specificities noted was obtained by Shack by means of fractional centrifugation and alcohol precipitation techniques, at low temperature, of kidney and of liver extracts (see p. 143) (54).

Chloroacetylglycyldehydroalanine is hydrolyzed at the same rate as is glycyldehydroalanine (Table I). Since no α -amino nitrogen is liberated in digests of the former, it is evidently hydrolyzed only at the dehydropeptide bond. The enzyme responsible is dehydropeptidase I, a fact still more clearly shown by the use of Shack's purified dehydropeptidase preparations (see tabulation on page 144). Since chloroacetylsarcosyldehydroalanine is not hydrolyzed, it would appear that the structural requirements of the substrate for dehydropeptidase I do not necessitate the presence of a free α -amino group adjacent to the dehydropeptide bond, but only the presence of an α nitrogen atom to which at least one hydrogen is attached. Dehydropeptidase II can dispense with this requirement although the presence of an α -chloro atom on the acyl residue makes the substrate more susceptible to the action of this enzyme. The presence of a β -phenyl group adjacent to the double bond in the dehydrophenylalanine peptides reduces the susceptibility of this class of compounds to the action of the dehydropeptidases. Differences in the activity ratios of the substrates in various tissues (Tables I and II) suggest the possibility that dehydropeptidases I and II may be further fractionated into subgroups.

The last point is emphasized by the resistance of chloroacetylglycyldehydrophenylalanine to enzymic attack, and by the totally different character of the pH-activity curves for alanyldehydroalanine and for chloroacetylalanyldehydroalanine (38a).

The structural requirements for a substrate for dehydropeptidase I are: $\text{RNHCH}_2\text{CONHC(=CH}_2\text{)COOH}$ (where R = acyl, alkyl, or hydrogen), and for dehydropeptidase II are: $\text{RCH}_2\text{CONHC(=CH}_2\text{)COOH}$ (where R = alkyl, halogen, or hydrogen) (52). The susceptibility of *N*-chloroacylated dehydropeptides suggests that dehydro-

peptidase I may be effective on dehydroamino acid residues at the terminal end of long-chain polypeptides, as in proteins.

The presence of dehydropeptidase I and II activity in human serum is illustrated in Table II. DL-Alanyldehydroalanine in particular is split to a considerable extent, and demonstrates the greater susceptibility of this substrate to the action of dehydropeptidase I than that of glycyldehydroalanine.

TABLE II
DEHYDROPEPTIDASE ACTIVITY IN HUMAN SERUM (38a,51)*

Subject	Ammonia nitrogen (micromoles) evolved from		
	DL-Alanyldehydroalanine	Glycyldehydroalanine	Chloroacetyldehydroalanine
M.L.E.	11.1	2.0	1.9
V.P.	10.8	2.0	2.0
C.D.	14.3	2.2	1.9
M.B.	10.5	2.0	1.9
W.D.	8.6	1.9	1.8
F.M.L.	10.8	2.1	2.0

* Digests consisted of 1 ml. serum + 2 ml. 0.15 *M* borate buffer at pH 8.1 + 1 ml. of either water or 25 micromoles of substrate (50 micromoles of DL-alanyldehydroalanine). Solutions of chloroacetyldehydroalanine neutralized with sodium hydroxide before use. Incubation period two hours at 37°C. No ammonia evolved under these conditions from digests of serum with glycine, glycylglycine, or DL-alanine. Chloroacetylglycyldehydroalanine is hydrolyzed at the same rate as glycyldehydroalanine, while chloroacetyl-DL-alanyldehydroalanine is not hydrolyzed at all (38a).

That the molar ratio of ammonia to pyruvic acid which appear in digests of tissue extracts with glycyldehydroalanine and chloroacetyldehydroalanine is close to unity was shown by Greenstein and Leuthardt (37), and is illustrated further by the experiments of Gonçalves, Price, and Greenstein (34) with acetyldehydroalanine (Table III).

It has been taken for granted that enzymic hydrolysis of the dehydropeptides was effected at the peptide bond, leading to the formation of the acyl acid and α -aminoacrylic acid. The latter hydrolyzes spontaneously to yield ammonia and pyruvic acid (III and IV, page 119). The possibility that the peptide might be split to yield the corresponding acid amide and α -keto acid was considered by Bergmann and Schleich (7) (XXV):



This possibility may however be rejected because under conditions whereby glycyldehydroalanine, chloroacetyldehydroalanine, and ace-

tyldehydroalanine readily yield ammonia in rat kidney digests, glycine amide yields very little ammonia, and chloroacetamide and acetamide yield no ammonia (34). The molar ratio of nearly unity for ammonia to pyruvate in digests of the dehydropeptides is definite proof for the splitting of these substrates at the peptide bond.

TABLE III
AMMONIA AND PYRUVIC ACID EVOLVED FROM ACETYLDEHYDROALANINE
IN DIALYZED EXTRACTS OF RAT TISSUES (34)*

Tissue	Ammonia, micromoles	Pyruvic acid, micromoles	Molar ratio, ammonia to pyruvic acid
Kidney	12.7	14.5	0.9
Liver	4.6	5.2	0.9

* Digests consisted of 1 ml. tissue extract equivalent to 333 mg. fresh tissue + 1 ml. veronal acetate buffer at pH 7.1, + 1 ml. of either water or 25 micromoles substrate. Period of incubation four hours at 37°C.

C. PURIFIED PREPARATIONS OF DEHYDROPEPTIDASE

Carter and Greenstein (14) achieved a sixfold concentration of dehydropeptidase activity toward glycyldehydroalanine through salt precipitation of aqueous extracts of rabbit kidney. Yudkin and Fruton (58) achieved a 5- to 10-fold concentration of activity toward glycyldehydrophenylalanine by a sodium sulfate precipitation of an aqueous extract of rat kidney. Prolonged dialysis of this preparation against demineralized water led to a diminution in activity which could be restored to the original value by addition of zinc salts (58). Treatment with cyanide or sulfide resulted in a significant inhibition of activity, and it would appear from these data of Yudkin and Fruton that the enzyme active in the hydrolysis of glycyldehydrophenylalanine belongs to the group of metal-containing enzymes.

By differential centrifugation at 3000 and 18,000 r.p.m., Shack (54) observed that the bulk of dehydropeptidase I of kidney is firmly bound to particulates sedimentable only at high speeds. In contrast, the dehydropeptidase I of liver and the dehydropeptidase II of liver and kidney remain in the supernate. A twentyfold concentration of dehydropeptidase I free of dehydropeptidase II was achieved by successive differential centrifugation, tryptic digestion, and salt fractionation of aqueous kidney extracts. The soluble enzymes were further purified by low-temperature alcohol fractionation, and the separation

of activities made in the fractionation procedures, as noted by the relative susceptibility of glycyldehydroalanine (substrate for dehydropeptidase I) and of chloroacetyldehydroalanine (substrate for dehydropeptidase II) confirmed the existence of these two distinct dehydropeptidases. Furthermore, as Shack noted, the activity ratio for each fraction when acetyldehydroalanine was used as substrate paralleled those when chloroacetyldehydroalanine was used as substrate, and it therefore can be assumed that both of these compounds serve as substrates for dehydropeptidase II.

Specificity studies by Shack utilizing various dehydropeptides with purified preparations from beef kidney are described in the accompanying tabulation. The data are consistent with those obtained with rat kidney extracts by Price and Greenstein (Table I).

SPECIFICITY STUDIES WITH PURIFIED DEHYDROPEPTIDASE PREPARATIONS (54)

Substrate	Dehydro-peptidase I	Dehydro-peptidase II
	Activity, micromoles substrate split/hour/mg. protein N	
Glycyldehydroalanine	5750	47.2
Glycyldehydrophenylalanine	2610	0
D,L-Alanyldehydroalanine	3900	22
Chloroacetyldehydroalanine	16.5	496
Chloroacetyldehydrophenylalanine	0	0
D,L- α -Chloropropionyldehydroalanine	0	0
Acetyldehydroalanine	0	334
Acetyldehydrophenylalanine	0	0
Chloroacetylglycyldehydroalanine	4650	58
Glycylglycyldehydroalanine	4500	42
Chloroacetylsarcosyldehydroalanine	0	0

Bergmann and Schleich (6) had noted in glycerol extracts of kidney that the hydrolysis of glycyldehydrophenylalanine was inhibited by cyanide. This finding was confirmed by Shack for the splitting of glycyldehydroalanine by purified dehydropeptidase preparations, and the additional observation was made that this inhibition was reversible, *i.e.*, simple dialysis of the cyanide-treated preparation against distilled water was sufficient to largely restore the activity. Dehydropeptidase II activity was also inhibited by cyanide and reversed by dialysis. The effect of several agents studied by Shack on the activity of the dehydropeptidases is given in the table below.

INHIBITION OF DEHYDROPEPTIDASES (54)

Agent	Dehydropeptidase I	Dehydropeptidase II
Sodium azide	—	—
Potassium iodide	—	—
Sodium fluoride	—	—
Sodium cyanide	+*	+*
Sodium thioglycolate	+*	+*
Sodium iodoacetate	—	+*

* Reversed on dialysis.

The pH-activity curves for purified preparations of the dehydropeptidases are given in Fig. 8. Yudkin and Fruton noted a pH optimum at 8.0 for glycyldehydrophenylalanine incubated with a rat kidney preparation (58).

The pH-activity curves for glycyldehydroalanine, chloroacetyl-glycyldehydroalanine, and alanyldehydroalanine closely resemble

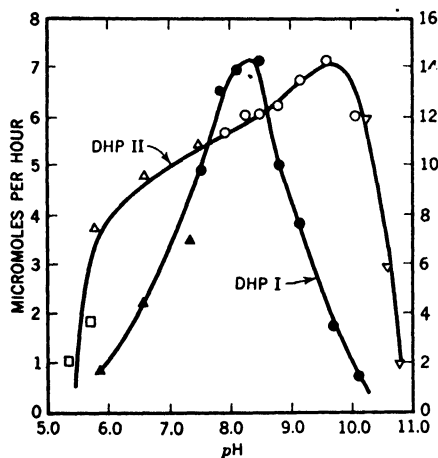


Fig. 8. pH-activity curves of purified dehydropeptidases at 38°C. (54). Ordinates: micromoles hydrolyzed per hour; on left, for dehydropeptidase I per 0.001 mg. enzyme N; on right, for dehydropeptidase II per 0.024 mg. enzyme N. O and ●, borate buffers; ▽, carbonate; Δ and ▲, phosphate; □ acetate. Substrate for DHP I, glycyldehydroalanine; for DHP II, chloroacetyldehydroalanine.

each other, but the curve for chloroacetylalanyldehydroalanine is almost identical with that for chloroacetyldehydroalanine (38a). The multiplicity of dehydropeptidases is suggested.

IV. Enzymic Degradation of Possible Precursors of Dehydropeptides

A. PEPTIDES OF CYSTINE

In 1939 Fromageot, Wookey, and Chaix (27) described an enzyme in liver which degraded cysteine with the formation of hydrogen

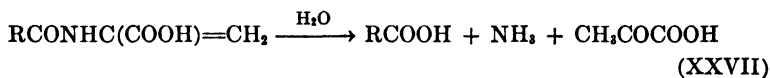
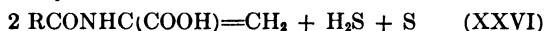
sulfide, and to which they gave the name *desulfurase*. Subsequently, Smythe (57) made an extensive study of this enzyme, in which he showed (a) that both cystine and cysteine were nearly equally degraded, whether under aerobic or under anaerobic conditions, and (b) that both substrates under these conditions yielded not only hydrogen sulfide but ammonia and pyruvic acid as well. Subsequent investigations by the writer confirmed these findings in all essential respects, and, as noted above, the rate at which various peptides of L-cystine, when incubated with liver extracts, yielded ammonia was very nearly the same as that for L-cystine itself (31,37) (Table IV).

TABLE IV
RATE OF SPLITTING OF AMMONIA NITROGEN FROM CYSTINE AND CYSTINE PEPTIDES
TREATED WITH RAT LIVER EXTRACTS (37)*

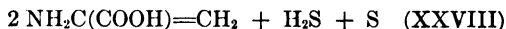
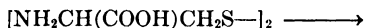
Time of incubation, min.	Ammonia N (micromoles) split off from				
	Cystine	Cystinyl-diglycine	Cystinyl-diglycine	Diglycyl-cystine	Dichloro-acetyl-cystine
30	4	4	6	4	
60	6	7	6	7	7
90	10	9		10	
120	13	12	11	13	14
150	15	15		15	
180	15	16	15	16	18

* Mixtures incubated two hours at 37°C. consisted of 2 ml. rat liver extract equivalent to 666 mg. fresh tissue + 1 ml. phosphate buffer at pH 7.0 + 1 ml. substrate solution at 25 micromoles concentration (on basis of half-molecule). Data corrected for extract blanks. Although dibromopropionylcystine is not enzymically attacked, dialanylcystine is split at the same rate as the substrates in Table IV (38).

Pyruvic acid also appeared in digests of all of the cystine peptides equivalent in concentration to the ammonia, and, for the reasons noted above, it seemed probable that the peptides of cystine are degraded in two consecutive steps: (XXVI) the enzymic desulfuration of the cystine moiety leading to free sulfur, hydrogen sulfide, and the corresponding α,β -dehydropeptide, followed by (XXVII) the hydrolysis of the dehydropeptide by dehydropeptidase to products which include pyruvic acid and ammonia in equivalent proportions.



These reactions, involving the peptides, may be considered analogous with the reaction applicable to the case of cystine itself, which also may be considered as proceeding by two consecutive steps: (XXVIII) the enzymic desulfuration to free sulfur, hydrogen sulfide, and α -aminoacrylic acid, the last-mentioned of which XXIX spontaneously hydrolyzes to ammonia and pyruvic acid.



Since glutathione is not readily split by rat liver tissue *in vitro*, the designation applied by Greenstein and Leuthardt (37) to the enzyme system which desulfurates the cystine peptides was *exocystine desulfurase*. Like dehydropeptidase, cystine desulfurase apparently acts only on the specific amino acid moieties when they are at the ends of the peptide chain (37).

Studies on a wide variety of tissues by Greenstein and Leuthardt (37) revealed that the distribution of exocystine desulfurase paralleled in remarkable fashion the distribution of the system which split chloroacetyldehydroalanine (dehydropeptidase II) (Table V).

It would appear from Table V, which largely represents a survey performed under optimal conditions, that exocystine desulfurase and dehydropeptidase II may be associated in tissues. The latter enzyme is found in appreciable activity only in liver and kidney, and only in these tissues is there also appreciable cystine desulfurase activity. In those tissues where one of these systems is lacking, so too is the other. The data in Table V, obtained under conditions whereby the susceptible substrates are nearly completely hydrolyzed, are not to be confused with the data in Table I.

Although sulfide inhibits dehydropeptidase activity in purified preparations of the enzyme (58), it has no effect on the hydrolysis of substrates in rat liver and kidney homogenates (38).

B. ISOMERIC PEPTIDES OF SATURATED AMINO ACIDS

On aerobic incubation of glycyl-DL-alanine and of DL-alanylglycine with aqueous homogenates of rat kidney tissue, it was noted that considerable ammonia accumulated in digests of the former peptide while little or no ammonia appeared in digests of the latter peptide

TABLE V. EXOCYSTINE DESULFHYDRASE AND DEHYDROPEPTIDASE ACTIVITY IN NORMAL AND NEOPLASTIC TISSUES (37)*

Tissue	Ammonia nitrogen evolved from									
	Cystine peptides ¹				Chloroacetyldehydroalanine ²					
	Rat	Mouse	Rab-bit	Guinea pig	Rat	Mouse	Rab-bit	Guinea pig	Rat	Mouse
Normal liver	11 ⁴	8	4	3	20 ^{1,5}	15	12	12	20 ^{1,5}	18
Regenerating liver	11	—	—	—	20	—	—	—	20	—
Transplanted hepatoma	0	0	—	—	1	0	—	—	20	18
Fetal liver	5	—	1	—	5	—	3	—	20	—
Primary hepatoma	0	—	—	—	10	—	—	—	20	—
Spleen	0	0	0	0	0	0	0	0	17	12
Kidney	4	3	3	2	20	23	14	16	22	21
Brain	0	0	0	0	0	0	0	0	17	15
Muscle	0	0	0	0	0	0	0	0	20	16
Pancreas	2	1	1	2	10	4	3	10	17	20
Intestinal mucosa	—	2	—	—	—	2	—	—	—	—
Intestinal adenocarcinoma	—	0	—	—	—	0	—	—	—	19
Melanoma	—	0	—	—	—	0	—	—	—	23
S37	—	0	—	—	—	0	—	—	—	21
CR180	—	0	—	—	—	0	—	—	—	20
Brown-Pearce tumor	—	0	0	—	—	0	0	—	—	17
Squamous-cell tumor	—	—	—	—	—	—	—	—	—	—
Fibrosarcoma	—	—	—	0	—	—	—	0	—	—
Jensen sarcoma	0	—	—	—	0	—	—	—	19	—
Lung tumor F	—	0	—	—	—	—	—	—	—	—
Blood serum ⁶	0	—	0	0	0	0	0	0	11	16
										14

* Data in terms of micromoles of ammonia nitrogen evolved from substrate after two hours of incubation at 38°C. Results corrected for blanks on tissue extracts.

¹ Cystine peptide substrates are, interchangeably, diglycylcystine and dichloroacetyl-cystine. Cystine yields identical results. Concentrations of these substrates based on half-molecules, e.g., 25 micromoles sulfur. Digestion mixtures composed of 1 ml. substrate, 1 ml. phosphate buffer at pH 6.9, and 2 ml. aqueous tissue extract equivalent to 333 mg. tissue per milliliter.

² Digestion mixtures composed of 1 ml. substrate, 1 ml. phosphate buffer at pH 6.9, and 2 ml. aqueous tissue extract equivalent to 333 mg. tissue per milliliter.

³ Digestion mixtures composed of 1 ml. substrate, 1 ml. phosphate buffer at pH 6.9, and 1 ml. aqueous tissue extract equivalent to 166 mg. tissue.

⁴ Pyruvate demonstrated.

⁵ Identical results obtained when digestion was conducted under anaerobic conditions.

⁶ Digests with serum conducted with 1 ml. undiluted and fresh material.

(35). Essentially similar findings were observed with isomeric peptides of DL-leucine (Table VI). Of further interest was the fact that

TABLE VI
AMMONIA NITROGEN PRODUCED FROM PEPTIDES AND AMINO ACIDS IN AEROBIC DIGESTS OF RAT KIDNEY HOMOGENATES (35) *

Substrate	Incubation, hr.	Ammonia N, micromoles †
DL-Alanine.....	4	6
	8	10
Glycyl-DL-alanine.....	4	6
	8	10
DL-Alanylglycine.....	4	<1
	8	1
DL-Leucine.....	4	5
	8	9
Glycyl-DL-leucine.....	4	5
	8	9
DL-Leucylglycine.....	4	<1
	8	1
L-Leucine.....	4	1
Glycyl-L-leucine.....	4	1
Glycine.....	8	0
Glycylglycine.....	8	0
DL-Valine.....	4	5
DL-Isovaline.....	4	0
DL- α -Amino- <i>n</i> -butyric acid.....	4	8
Glycyl-DL- α -amino- <i>n</i> -butyric acid.....	4	8
α -Aminoisobutyric acid.....	4	0
Glycyl- α -aminoisobutyric acid.....	4	0
DL-Leucylglycylglycine.....	4	0
Glycyl-DL-leucylglycine.....	4	0
Glycylglycyl-DL-leucine.....	4	5
D-Leucylglycine.....	4	0
D-Leucylglycine + 0.001 <i>M</i> MnCl ₂	4	1
D-Leucylglycine + Mn + L-leucine.....	4	<1
D-Leucylglycylglycine.....	4	0
D-Leucylglycylglycine + 0.001 <i>M</i> MnCl ₂	4	0

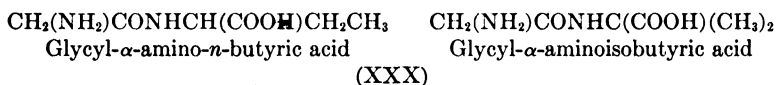
* Digests consisted in 1 ml. dialyzed homogenate equivalent to 333 mg. tissue + 2 ml. 0.15 *M* borate buffer at pH 8.1 + 1 ml. 0.05 *M* racemic or 0.025 *M* optically active substrate. Enzymic activity was measured by the amount of ammonia produced corrected for the extract blanks. No ammonia was produced from any substrate when the digestion was conducted under anaerobic conditions. Temperature 37°C.

† Theoretical maximum 25 micromoles from each optically active component.

the ammonia which appeared in digests of glycyl-DL-alanine and of glycyl-DL-leucine was close in order of magnitude to that which appeared in digests of DL-alanine and of DL-leucine, respectively.

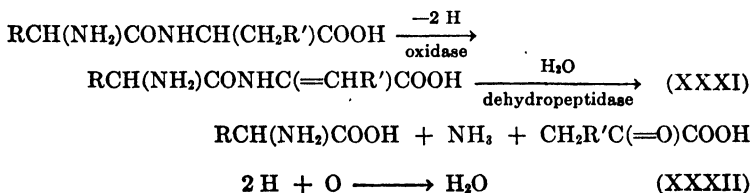
The ammonia which appears from the racemic substrates under

these experimental conditions is due principally to D-amino acid oxidase activity, and may be related specifically to the oxidative deamination of the D-amino acid moiety of the peptides (42). Neither L-leucine nor glycyl-L-leucine yields appreciable ammonia under these conditions. That the oxidative deamination involves the α and β hydrogen atoms of the substrate is revealed in the relative susceptibility of glycylamino-*n*-butyric acid and of glycylaminoisobutyric acid (Table VI). The latter possesses a tertiary carbon atom in the isobutyric acid residue. The results on valine and isovaline may be interpreted similarly.



The contribution of the L-amino acid components to the yield of ammonia in digests of the racemic, isomeric peptides may therefore be relatively neglected, and the role of the natural L-peptidase in the splitting of the L form of the peptides in the kidney digests is not of immediate concern in the interpretation of the phenomena. This leaves for consideration only the D form of the peptides, namely, D-alanylglycine and D-leucylglycine on the one hand, and glycyl-D-alanine and glycyl-D-leucine on the other. Two alternative explanations for the behavior of the isomeric peptides may be offered.

(1) D-Amino acid oxidase may be considered as acting only on free D-amino acids, and therefore the ammonia noted in digests of the racemic peptides could only have arisen subsequent to the action of D-peptidase on the peptides liberating the free amino acids. On this basis, it would appear that glycyl-D-alanine and glycyl-D-leucine were very susceptible, whereas D-alanylglycine and D-leucylglycine were very resistant, to the action of D-peptidase. This would not be in agreement with the relative susceptibility of the corresponding L-peptides to intestinal dipeptidase, by which alanylglycine is hydrolyzed at twice the rate as glycylalanine (43). (2) D-Amino acid oxidase may be considered as acting not only on the free D-amino acids, but also upon D-amino acids bound through the amino group in peptide linkage with another amino acid. Such a concept is in harmony with Bergmann's view that the oxidative deamination of amino acids might be ef-



fected while they are in the peptide chain, yielding by an α,β -dehydrogenation the corresponding dehydropeptide, which subsequently is split by dehydropeptidases to products which include ammonia and keto acids (see equations XXXI, XXXII).

On this basis, dipeptides which contain a glycine residue at the carboxyl end of the chain, as in alanyl glycine and leucyl glycine, could not form dehydropeptides whereas peptides like glycylalanine and glycylleucine could form such α,β -unsaturated peptides. The data on the three isomeric tripeptides of DL-leucine and glycine are consistent with this viewpoint. Kidney tissue is, of all animal tissues, richest in both D-amino acid oxidase (42) and dehydropeptidase (14).

The second of these alternatives was sympathetically considered by Krebs in his early work on the subject of amino acid oxidation, but no decision was reached by him (42). Krebs's data reveal that L-leucyl glycine, glycyl-L-leucine, or D-leucyl glycine when incubated with rat kidney slices yields little if any ammonia. The data of Gonçalves, Price, and Greenstein (35) (Table VI) obtained with rat kidney homogenates are consistent with these results. Unfortunately, Krebs did not test glycyl-D-leucine.

On the basis of the present data it is possible to favor this second alternative, which is not only consistent with the Bergmann concept of intracellular peptide metabolism, but also supplements the earlier work on the enzymic susceptibility of peptides of L-cystine (37) (Table V, page 148).

TABLE VII
ENZYMIC SUSCEPTIBILITY OF ANALOGOUS SATURATED AND UNSATURATED PEPTIDES

Saturated peptides	Ammonia N*	Unsaturated peptides	Rate†
Acetyl-DL-alanine	0.0	Acetyldehydroalanine	18
Chloroacetyl-DL-alanine	0.0	Chloroacetyldehydroalanine	100
Glycyl-DL-alanine	6.0	Glycyldehydroalanine	1620
Acetyl-DL-phenylalanine	0.0	Acetyldehydrophenylalanine	0
Chloroacetyl-DL-phenylalanine	0.0	Chloroacetyldehydrophenylalanine	0
Glycyl-DL-phenylalanine	6.2	Glycyldehydrophenylalanine	520
DL-Alanine	6.1		
DL-Phenylalanine	6.0		

* Ammonia nitrogen in micromoles above extract controls which appears in aerobic digests consisting of 1 ml. rat kidney extract equivalent to 333 mg. fresh tissue + 2 ml. 0.15 M borate buffer at pH 8.1 + 1 ml. of either water or 0.05 M DL substrate. Ammonia evolved from the L form of the amino acids negligible, and from glycine or glycyl glycine, zero (35). No ammonia observed in digests under anaerobic conditions. Acetyl- and chloroacetylpeptides neutralized with NaOH before use. Period of incubation four hours at 37°C.

† Data from Table I.

A comparison of the relative susceptibility to enzymic attack based on considerations of molecular configuration is given for analogous saturated and unsaturated peptides in Table VII. For enzymic attack on the saturated peptides of alanine and of phenylalanine, and on the unsaturated peptides of dehydrophenylalanine, the presence of a free α -amino group on the acyl residue is apparently necessary. For the unsaturated peptides of dehydroalanine, no such requirement is necessary.

C. DIACYLAMINOPROPIONIC ACIDS

The dehydropeptides of α -aminoacrylic acid (α,β -dehydroalanine) may be considered essentially a combination of one mole of pyruvic acid with one mole of the corresponding acid amide with the elimination of the elements of water between them (3) (see XIV, page 124). When the acyl residue contains an α -amino group as in glycyldehydroalanine, the dehydropeptide is susceptible to the action of an enzyme which is widespread and highly active in all animal and plant tissues studied, which splits the substrate into products which include pyruvic acid and ammonia in equimolar proportions, and to which the designation dehydropeptidase I has been applied (37) (see page 119).

The possibility has been pointed out that the dehydropeptides may actually be formed in tissues by a condensation between α -keto acids and amino acid amides (1,36), and the results of experiments on the effect of pyruvate on the rate of deamidation of such naturally occurring amides as glutamine and asparagine have been interpreted on this basis (see page 124) (36).

Pyruvic acid, however, can condense not only with one but also with two moles of acid amides, forming α,α -diacylamino propionic acid peptides (3,32,44,47) (see XIV and XVII, pages 124 and 126). The reversibility of the conversion of the diacylamino propionic acids into the corresponding dehydropeptides plus acid amide—*in vitro*—has been described in a previous section; see XXI, page 128. The possibility as to whether this conversion may occur *in vivo* was examined by Gonçalves and Greenstein in their studies on α,α -diglycylamino propionic acid hydrochloride (32) (XX).

Glycyldehydroalanine, by virtue of its capacity for existing in several tautomeric forms, possesses a characteristic absorption in the ultraviolet region of the spectrum, with a maximum at 2400 Å (14) (Fig. 2, page 131). Other aliphatic dehydropeptides have a similar

absorption (51). Diglycylaminopropionic acid may be considered to be glycyldehydroalanine in which the double bond has been saturated by the substitution of a molecule of glycine amide. The absorption characteristics of the dehydropeptide are no longer present, and the spectrum of diglycylaminopropionic acid reveals only a general absorption in the ultraviolet (32). The electrochemical properties of the peptide have been referred to above.

Diglycylaminopropionic acid is readily hydrolyzed into products which include equimolar proportions of ammonia and pyruvic acid in homogenates of rat tissues (Table VIII). Kidney tissue is particularly active. Under similar conditions, no increase in ammonia or pyruvic acid over the values for the controls was noted in digests of dichloroacetaminopropionic acid or of diacetaminopropionic acid.

TABLE VIII

AMMONIA NITROGEN AND PYRUVIC ACID EVOLVED FROM DIGESTS OF DIGLYCYLAMINOPROPIONIC ACID WITH DIALYZED RAT TISSUE HOMOGENATES (32)*

Tissue	Ammonia N, micromoles	Pyruvic acid, micromoles	Molar ratio, ammonia N to pyruvic acid
Kidney†	23	21	1.1
Intestinal mucosa	13	14	0.9
Liver	9	9	1.0
Spleen	5	6	0.8

* Digests consisted of 1 ml. dialyzed homogenate equivalent to 333 mg. tissue + 2 ml. 0.15 *M* borate buffer at pH 8.1 + 1 ml. of either water or 25 micromoles of substrate. Incubation period, four hours at 37°C. Final pH 7.9 to 8.0. Values given corrected for extract blanks. Recovery experiments for ammonia yielded 95–100% and for pyruvic acid 90–95%. Micromoles of ammonia N in digests with pancreas 11, with brain 1, and with muscle 1. No reaction occurs with boiled homogenate preparations.

† Neither glycine nor glycylglycine yields ammonia under these conditions.

The maximum rate of splitting of diglycylaminopropionic acid occurs at pH 8.0, and at each pH value of the digests studied the molar ratio of ammonia to pyruvic acid is close to unity (32) (Fig. 9). Furthermore, the maximum number of moles of ammonia nitrogen or pyruvic acid formed per mole of substrate is close to unity, and this relation apparently holds over the entire time interval investigated (32) (Fig. 10).

High-speed centrifugation at low temperature of aqueous rat kidney extracts yields a pellet in which the greater part of the activity

of the extract is concentrated. However, in each of the fractions tested, the molar ratio of ammonia nitrogen to pyruvic acid remains close to unity (Table IX).

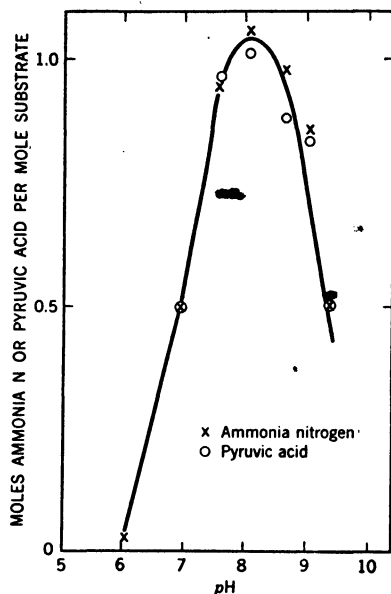


Fig. 9. Ammonia and pyruvic acid split from α, α -diglycylaminopropionic acid in rat kidney digests at different pH values (32).

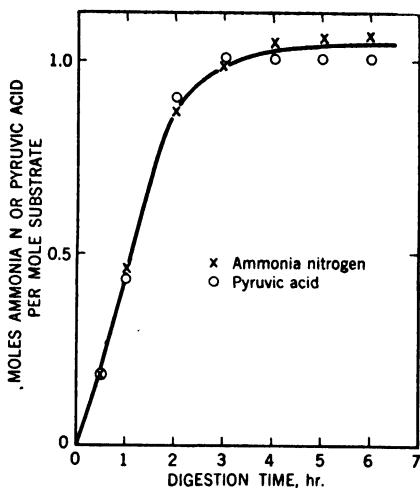


Fig. 10. Progressive splitting of α, α -diglycylaminopropionic acid into ammonia and pyruvic acid in rat kidney digests at pH 8.0 (32).

TABLE IX

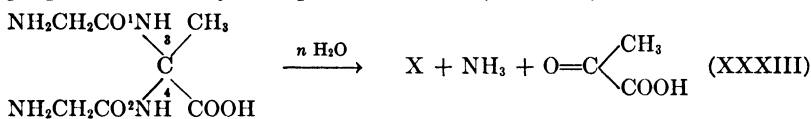
DISTRIBUTION OF ACTIVITY IN HYDROLYSIS OF DIGLYCYLAMINOPROPIONIC ACID IN FRACTIONS OF RAT KIDNEY EXTRACT CENTRIFUGED FOR TWO HOURS AT 5°C. AND AT 18,000 REVOLUTIONS PER MINUTE (32)*

Fraction	Ammonia N, micromoles	Pyruvic acid, micromoles	Molar ratio, ammonia N to pyruvic acid
Supernatant	8.3	8.2	1.0
Pellet suspended in distilled water†	22.4	21.6	1.0
Pellet resuspended in supernatant	21.0	19.8	1.0

* Experimental conditions as in Table VIII.

† Suspension at same volume as original extract.

The molar ratio of ammonia to pyruvic acid formed in the course of the enzymic splitting of diglycylaminopropionic acid is important in the interpretation of the mode of such splitting. The maximum amount of pyruvic acid that can be formed from the complete splitting of one mole of diglycylaminopropionic acid is obviously one mole. Depending upon the mode of splitting, however, the maximum ammonia nitrogen produced may be either one or two moles per mole of peptide, and the ratio of ammonia nitrogen to pyruvate formed during the course of the splitting may suggest not only the mode of splitting but also how many modes may be involved. The α -amino groups on the glycyl residues are presumably not hydrolyzable and do not furnish ammonia. Neither glycine nor glycylglycine furnishes ammonia after digestion with tissue extracts. The ammonia nitrogen is furnished by the nitrogen atoms attached to the tertiary carbon atom of the peptide, and on complete hydrolysis in boiling hydrochloric acid two moles of ammonia nitrogen are formed per mole of peptide (32). Before pyruvic acid can be formed from the substrate, both linkages from the diglycylamino residues to the tertiary carbon must obviously be broken. The modes of splitting of diglycylaminopropionic acid may be represented as in (XXXIII).



(a) The substrate may be initially split at both peptide bonds, 1 and 2, yielding two moles of glycine, and one mole of α, α -diaminopropionic acid. The last-mentioned compound is unstable and would be expected to break down spontaneously to two moles of ammonia nitrogen and one mole of pyruvic acid. Molar ratio of ammonia nitrogen to pyruvate = 2. (b) The substrate may be initially split at only one peptide bond, either 1 or 2, yielding one mole of glycine and one mole of α -glycylamino- α -aminopropionic acid. The latter, in analogy with α -diacetamino- α -aminopropionic acid (XVIII, page 126), would be expected to be unstable and to break down spontaneously into one mole of glycine amide, one mole of ammonia, and one mole of pyruvic acid. Provided that the ammonia nitrogen contributed by the enzymic deamidation of the glycine amide were negligible, the molar ratio of ammonia nitrogen to pyruvate = 1. (c) The substrate may be initially split at both bonds 3 and 4 which con-

nect the glycylamino residues to the tertiary carbon atom, yielding two moles of glycine amide and one mole of pyruvic acid. If the glycine amide is completely and rapidly split, the molar ratio of ammonia nitrogen to pyruvate = 2. (d) The substrate may be initially split at only one bond between nitrogen and the tertiary carbon atom, that is, at either 3 or 4, yielding glycine amide and glycyldehydroalanine. Provided that the ammonia nitrogen contributed by the enzymic deamidation of the glycine amide is negligible, and, since glycyldehydroalanine is rapidly hydrolyzed by dehydropeptidase I to yield equimolar amounts of ammonia and pyruvic acid, the molar ratio of ammonia nitrogen to pyruvate = 1.

Under the experimental conditions employed, diglycylaminopropionic acid hydrochloride is rapidly hydrolyzed to yield a maximum of one mole of ammonia nitrogen and of one mole of pyruvic acid per mole substrate, and therefore the molar ratio of ammonia nitrogen to pyruvate = 1. Modes of splitting *a* and *c* may be eliminated from consideration, leaving the choice between modes *b* and *d*. The deamidation of glycine amide is weak, and may for present purposes be neglected (32). Mode *b* envisages the action of some enzyme related to or identical with dipeptidase on one of the two peptide bonds, leading to the formation of an unstable molecule, which spontaneously hydrolyzes to products that include equimolar amounts of ammonia and pyruvic acid. Kidney extracts are known to contain an active dipeptidase, but intestinal mucosa and pancreas contain a still more powerful dipeptidase activity, and it may be wondered why the activity of kidney extracts in splitting diglycylaminopropionic acid is so much greater than that of either intestinal mucosa or pancreas. Another possible objection to mode *b* is the fact that peptides of glycine with amino acids containing a tertiary carbon atom, such as glycylaminoisobutyric acid, are relatively resistant to the action of dipeptidase (11). According to Bergmann *et al.*, substrates for dipeptidase must have a hydrogen atom on each α carbon atom adjacent to the peptide bond, and neither diglycylaminopropionic acid nor glycylaminoisobutyric acid, nor, for that matter, glycyldehydroalanine, satisfy this criterion. None of these three peptides possesses an asymmetric carbon atom. On the other hand, it should be pointed out that diglycylaminopropionic acid is a type of substrate new in enzymology, and the possibility cannot be overlooked that crude tissue extracts may contain an enzyme system different from

the classical dipeptidase system which may readily attack this substrate at one of the two peptide bonds. Assuming the existence of such a system, it is possible that it attacks both peptide bonds simultaneously since both bonds are presumably equivalent. Were this to occur, the molar ratio of ammonia nitrogen to pyruvate would = 2 (mode *a*).

Mode *d* envisages the action of an enzyme system not known at the present time to occur in tissues, which presumably splits glycine amide from the substrate and which leaves glycyldehydroalanine to yield equimolar quantities of ammonia and pyruvic acid through the action of dehydropeptidase I. Such a reaction would be the biological analog of the *in vitro* reactions whereby diacylamino propionic acids are split into the corresponding acid amide and dehydropeptide through the action of hot glacial acetic acid (3,44,47) (see XV and XXI, pages 124 and 128). The *in vitro* experiments are further suggestive in revealing that the diacylamino radical in the diacylamino propionic acid peptides are not necessarily equivalent, and that the strength of the bonds holding them to the tertiary carbon atom may be greater in one than in the other.

At the present time it is impossible to make an absolute choice between modes *b* and *d* in the enzymic splitting of diglycylamino propionic acid. The possibility that both may simultaneously occur is of course not excluded. Whatever the nature of the enzyme systems may be which provide the initial attack upon diglycylamino propionic acid, it is evident that the substrate upon which they act must possess free α, α -diamino groups on the molecule, since the corresponding α, α -dichloroacetaminopropionic acid and α, α -diacetaminopropionic acid are not split under conditions whereby the α, α -diamino compound is rapidly hydrolyzed (32).

Compounds of this type containing asymmetric carbon atoms have been prepared and studied by Price, Errera, and Greenstein (53). α, α -Di(DL-chloropropionylamino)propionic acid was not affected by rat kidney extracts. α -(DL-Chloropropionylamino)- α -(DL-alanyl-amino)propionic acid was hydrolyzed by rat kidney extracts to a maximum of 0.5 mole of ammonia nitrogen and 0.5 mole of pyruvic acid per mole of substrate: at each time interval studied the molar ratio of ammonia to pyruvic acid was close to unity. The product obtained by further amination, namely, α, α -di(DL-alanyl-amino)propionic acid hydrochloride, was also enzymically hydrolyzed but, at

each time interval studied, the molar ratio of ammonia nitrogen to pyruvic acid was close to 1.5. Each of these compounds is a mixture of optical enantiomorphs, and the interpretation of the data is rendered difficult because of this fact.

D. EFFECT OF α -KETO ACIDS ON DEAMIDATION OF GLUTAMINE AND ASPARAGINE

The deamidation of glutamine and of asparagine in liver digests is considerably augmented by added pyruvic acid at a pH (6.7 to 6.8) at

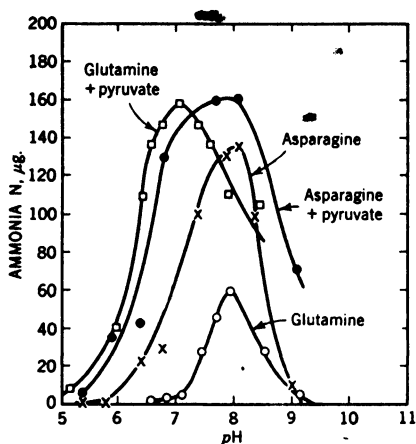


Fig. 11. pH-activity curves of amides alone and with pyruvate in rat liver digests: amides, 14 micromoles; pyruvate 23 micromoles. The incubation period was four hours at 37°C. (30).

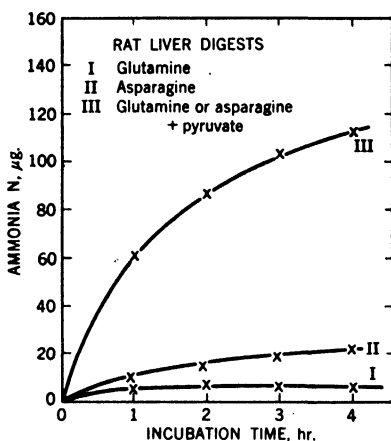


Fig. 12. Time course of deamidation of glutamine and asparagine with and without added pyruvate in rat liver digests pH 6.8: amides, 14 micromoles; pyruvate, 23 micromoles (50).

which glutaminase activity is negligible and asparaginase activity well below the maximum (15,22,23,26,31,33,36,50) (Figs. 11 and 12). The ammonia which appears in such digests can be quantitatively accounted for by a corresponding decrease in the amide nitrogen of the amides, and the added pyruvic acid is nearly completely recovered at the end of the incubation period (26,36). The pyruvic acid apparently plays the role of a cosubstrate in the deamidation reaction.

The phenomenon appears to be limited to glutamine, asparagine, and chloroacetylglutamine of all the amides tested, for the deamida-

tion in liver digests of isoglutamine, chloroacetylglutamine, glycyl-asparagine, and benzoylarginine amide is not enhanced by added pyruvic acid (33,38,50). Furthermore, only liver, of all animal tissues studied, possesses the capacity of augmenting the deamidation of glutamine and of asparagine (38,50).

The pyruvic acid need not be added as such, but may be derived from the simultaneous enzymic degradation of other compounds, such as dehydropeptides of α -aminoacrylic acid or peptides of L-cystine (31). Thus, the deamidation of glutamine in liver digests

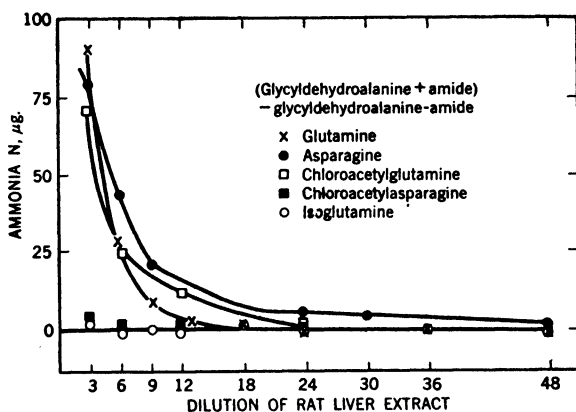


Fig. 13. Differences in ammonia values between digests containing glycyldehydroalanine plus amide and the sum of glycyldehydroalanine and of the amide taken separately, as a function of the dilution of rat liver extract (33).

containing either glycyldehydroalanine or diglycyl-L-cystine is augmented to nearly the same degree as it would be were the equivalent amount of pyruvic acid added equal to that derived from the breakdown of these peptides (Table X) (Fig. 13). The gist of the data in Table X relates to the fact that, in dialyzed extracts of rat liver in which the degradation of diglycyl-L-cystine is very considerably decreased (37), no increase in ammonia nitrogen is noted in the presence of glutamine. When pyruvate is added to such a digest of the dialyzed extract with glutamine, a considerable increase in ammonia nitrogen production results. Under the conditions used, all exocysteine peptides studied had substantially the same effect (31).

TABLE X
DEAMIDATION OF GLUTAMINE IN DIALYZED AND UNDIALYZED RAT LIVER
EXTRACTS IN ABSENCE AND PRESENCE OF DIGLYCYL-L-CYSTINE (31)*

Ammonia N (micromoles) observed in								Increase in micromoles ammonia N in digests of glutamine + peptide over sum of both	
Extract alone		Extract + glutamine		Extract + peptide		Extract + glutamine + peptide			
Dialyzed	Undialyzed	Dialyzed	Undialyzed	Dialyzed	Undialyzed	Dialyzed	Undialyzed	Dialyzed†	Undialyzed
0	3.0	1.6	3.4	2.0	12.5	3.6	18.7	0.0	5.8

* Fresh aqueous rat liver extract equivalent to 333 mg. tissue per ml. was divided into halves, and one half was dialyzed against distilled water at 5° for 24 hours. Digests consisted in 1 ml. extract + 1 ml. glutamine containing 2.1 mg. + 1 ml. diglycylcystine containing 10 mg., with water alone in place of the substrate solutions where desired. Mixtures digested six hours at 37°C.

† When 3.2 mg. sodium pyruvate is substituted for diglycylcystine, the increase in deamidation of the glutamine amounts to 6.6 micromoles.

The augmenting effect of pyruvic acid on the deamidation of glutamine and of asparagine can be separated from the respective glutaminase and asparaginase activities not only by the fact that these enzymes are more acid labile (Fig. 11) (23) but also more heat labile (23) (Fig. 14). If the liver extracts are kept at pH < 6 or at a temperature of 50°C. for ten minutes before, respectively, neutralizing or cooling, mixing with the substrates, and digesting at pH 8 and at 37°C., the capacity of such extracts to deaminate glutamine or asparagine alone is lost, but not the capacity to deaminate glutamine or asparagine in the presence of pyruvic acid (23). The enzyme systems responsible for the deamidation of these amino acid amides in the presence of pyruvic acid are evidently not identical with glutaminase or asparaginase.

Of the keto acids studied, only pyruvate, phenylpyruvate, and, α -ketoisocaproate increase the deamidation of glutamine in liver digests (26,52a) (Fig. 15). The effect of pyruvic acid reaches a maximum at a ratio of 2-3 moles per mole of glutamine (for 2 concentrations of glutamine), while the effect of phenylpyruvic acid reaches a maximum at a ratio of 1 mole per mole of glutamine. Levulinic acid, pyruvoylglycine, phenylpyruvoylglycine, and α -ketoglutaric acid have little or no effect on the deamidation of glutamine. That the effect of pyruvic acid is due essentially to the presence of the carbonyl group is shown by the fact that lactic acid has a relatively negligible

effect (Fig. 15). Whether the added keto acids increased or did not increase the deamidation of glutamine, they nevertheless could be nearly quantitatively recovered at the end of the incubation as the respective 2,4-dinitrophenylhydrazones (26,52a).

These results were originally interpreted by Greenstein and Carter (36), and subsequently sustained (15,22,23,26,31,33,50) on the basis

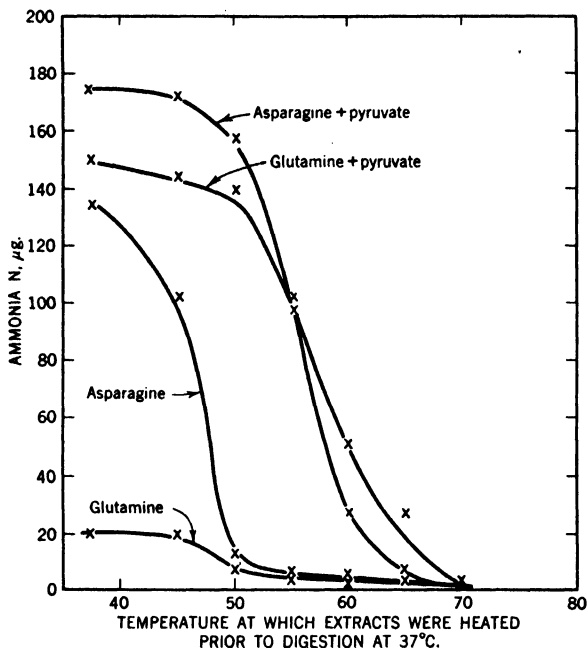


Fig. 14. Amide nitrogen as ammonia split at pH 8.0 from digests of glutamine and asparagine alone and in the presence of added pyruvate with aqueous rat liver extracts which had been heated for ten minutes at various temperatures prior to digestion of the substrates at 37°C. (23).

of the analogy to the *in vitro* reaction of Bergmann and Grafe (3) (XIV, page 124) whereby amides readily condense with pyruvic acid to form dehydropeptides. On this basis, the deamidation of glutamine or asparagine may be considered to occur in two consecutive steps: XXXIV, a condensation between the amide group of glutamine or asparagine with the carbonyl group of pyruvic acid to

form a dehydropeptide, followed by hydrolysis (XXXV) of the dehydropeptide to glutamic or aspartic acid, ammonia, and the regenerated pyruvic acid. The proposed condensation reaction

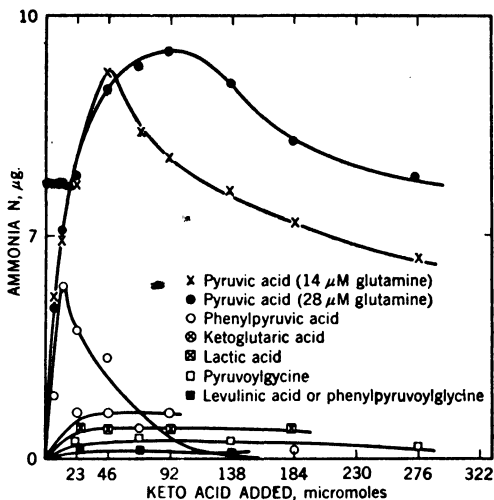
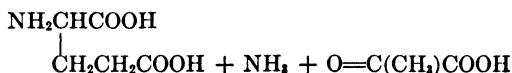
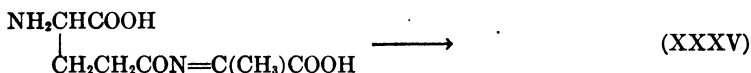
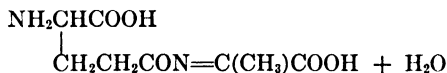
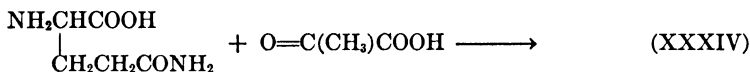


Fig. 15. Effect of added keto acids and lactic acid on the deamidation of glutamine at pH 6.8 in rat liver extracts. Ordinate refers to increment over glutaminase activity (usually <0.5 micromole). Incubation period four hours at 37°C. (26).

(XXXIV) involves the γ group of glutamic acid, a union noted physiologically in glutathione.



Increasingly large amounts of added pyruvic acid or phenylpyruvic acid appear to diminish the accelerating effect on the deamidation

of glutamine (Fig. 15), while pyruvoylglycine and phenylpyruvoylglycine have no effect on glutamine deamidation at any concentration studied. If there is any truth to reactions XXXIV and XXXV, it would be expected that at high concentrations pyruvic acid and phenylpyruvic acid would inhibit dehydropeptidase activity, whereas their peptides with glycine would not inhibit appreciably. This was found to be the case (26) (Table XI). The curves in Figure 15 relating to pyruvic acid and to phenylpyruvic acid may be a composite of two curves. The ascending portion may be due to the increased rate of condensation of pyruvic acid or phenylpyruvic acid with glutamine as the keto acid concentration is increased to a point where the large amount of added keto acid produces such an inhibition of dehydropeptidase activity (Table XI) as to overcome its accelerating effect on the initial condensation reaction, with the result that the curve begins to descend. On this basis, the keto acid peptides, which do not accelerate the deamidation of glutamine appreciably do not condense with the glutamine, nor do they inhibit dehydropeptidase activity.

TABLE XI

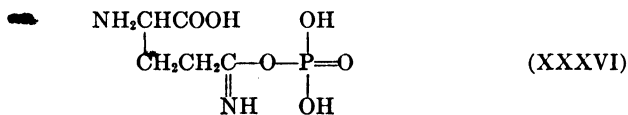
EFFECT OF KETO ACIDS AND KETO ACID PEPTIDES ON HYDROLYSIS OF DEHYDROPEPTIDES (26)*

Keto acid or keto acid peptide added	Ammonia evolved (micromoles) from		Per cent inhibition of the splitting of	
	Glycyl-dehydro-alanine	Glycyldehydrophenyl-alanine	Glycyl-dehydro-alanine	Glycyldehydrophenyl-alanine
	6.3	1.3		
Pyruvic acid	4.2	0.0	34	100
Phenylpyruvic acid	1.2	0.9	81	31
Pyruvoylglycine	6.0	1.2	5	7
Phenylpyruvoylglycine	6.0	1.2	5	7

* Digests consisted of 1 ml. aqueous rat liver extract equivalent to 333 mg. fresh tissue + 1 ml. veronal acetate buffer at pH 6.8 + 1 ml. dehydropeptide solution containing 23 micromoles substrate + 1 ml. of either water or keto acid solution containing 276 micromoles. Keto acid solutions at pH 7.0. Period of incubation when glycyldehydroalanine was used one hour at 5°C. and when glycyldehydrophenylalanine was used, two hours at 37°C. Data corrected for extract blanks. pH at end of experiments 6.7 to 6.8.

The deamidation of glutamine is accelerated by a number of inorganic anions, preeminently by phosphate, but also by arsenate and sulfate anions (15). The optimum pH for this effect is identical

with that of glutaminase. It would appear that this effect was primarily that of an activation of glutaminase, for, when this enzyme was inactivated by acid or heat treatment, subsequent addition of phosphate, unlike that of pyruvate, did not produce an appreciable deamidation of glutamine (23). The possibility, however, cannot be excluded that the activation of the deamidation of glutamine may be due to a reaction with the substrate, producing a highly labile phosphate ester (XXXVI) of the imino form of the amide (15):



The numerous differences between the phenomena of pyruvate and of phosphate acceleration of glutamine deamidation have been described (38). Thus, whereas glutamine deamidation is accelerated by pyruvate only in liver, it is accelerated by phosphate in liver, brain, and spleen.

V. Dehydropeptidase I Activity in Tumors and in Pathological Sera

Dehydropeptidase I activity in tumors is considerably higher than that of corresponding normal tissues (14). Studies by Meister and Greenstein (45) on human pathological sera revealed that significant elevations in serum dehydropeptidase activity occurred in cases of liver disease (Fig. 16).

VI. General Aspects

Several enzyme systems are known in tissues which act upon the $\text{C}(=\text{O})-\text{N}$ bond, among them the wide variety of proteases, peptidases, urease, and the dehydropeptidases. While the bond itself is the ultimate object of the enzymic attack, the molecular configuration about this bond determines the susceptibility of the bond to specific enzymes and their rate of action. Thus, for the action of proteases and peptidases, (a) the nitrogen in the bond must have a hydrogen attached, and (b) both carbon and nitrogen must be attached to a carbon atom. Prolinase apparently dispenses with a, and the nitrogen in the susceptible linkage is attached by a bond to each of two carbon atoms. In the case of urease, the carbon in the vulnerable

C(=O)—N bond is attached to another nitrogen atom. Dehydropeptidase differs from all these systems in requiring for its ultimate

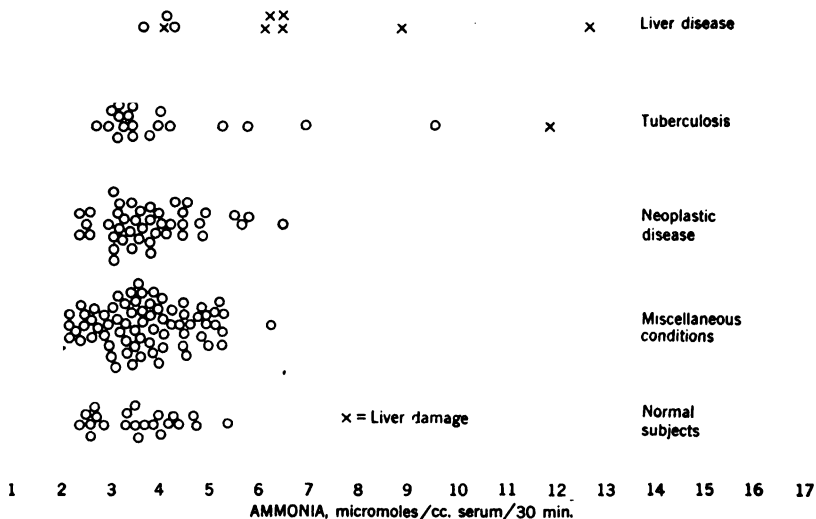


Fig. 16. Dehydropeptidase activity (DL-alanyldihydroalanine as substrate) in normal and pathologic human sera (45). Maximum hydrolysis equivalent to 25 micromoles ammonia.

action in addition to a double bond in which the carbon attached to the nitrogen participates. These may be represented as follows:

Enzyme	Susceptible linkage
Proteases and peptidases.....	—C—C(=O)NHC—
Dehydropeptidases.....	$\text{—C—C(=O)NHC(=C)—C—}$ $\text{—C—C(=O)N=C(—C)—C—}$
Prolinase.....	—C—C(=O)N C—C— C—C—
Urease.....	—N—C(=O)—N—

Beyond the nature of the susceptible linkage, the individual enzymes concerned require additional specific groups attached to this linkage for their action to be effective. Thus, the action of dipepti-

dase requires the presence of a free α -amino group, a free α -carboxyl group, and hydrogen atoms attached to the two carbon atoms combined at either side of the susceptible linkage, whereas, for the action of carboxypeptidase, the absence of the free α -amino group is essential but the other requirements for the susceptible substrate must be met. The molecular configurations essential for dehydropeptidase activity bear some resemblances to these, thus, for the action of both dehydropeptidase I and dehydropeptidase II, the presence of an α -carboxyl group in the substrate is required, but whereas for dehydropeptidase I the presence of an α -nitrogen atom with at least one hydrogen is needed, for dehydropeptidase II the absence of this group is required. The substrate configuration for dipeptidase and dehydropeptidase I is somewhat analogous, as is that for carboxypeptidase and dehydropeptidase II:

Enzyme	Susceptible configuration
Dipeptidase.....	$-\text{CH}(\text{NH}_2)\text{C}(=\text{O})\text{NHCH}-\text{COOH}$
Dehydropeptidase I.....	$-\text{CH}(\text{NHX})\text{C}(=\text{O})\text{NHC}(=\text{CHR})-\text{COOH} \rightleftharpoons$ $-\text{CH}(\text{NHX})\text{C}(=\text{O})\text{N}=\text{C}(-\text{CH}_2\text{R})-\text{COOH}$
Carboxypeptidase.....	$-\text{CH}(\text{R})\text{C}(=\text{O})\text{NHCH}-\text{COOH}$
Dehydropeptidase II....	$-\text{CH}(\text{R})\text{C}(=\text{O})\text{NHC}(=\text{CHR})-\text{COOH} \rightleftharpoons$ $-\text{CH}(\text{R})\text{C}(=\text{O})\text{N}=\text{C}(-\text{CH}_2\text{R})-\text{COOH}$
where R = hydrogen, hydroxyl, halogen, or $-\text{C}(=\text{O})-\text{N}-$ X = hydrogen, methyl, or CH_3CO	

These analogies hold only for the nature of the susceptible configurations. When the rate of splitting of the susceptible $-\text{C}(=\text{O})-\text{N}-$ linkage is considered, certain differences emerge. Thus, the action of carboxypeptidase is considerably accelerated if a phenyl group is substituted on the α -carbon atom to which the free carboxyl group is attached, but this substitution on the other hand inhibits the action of dehydropeptidase II, probably by reducing the $\text{C}=\text{C}$, $\text{C}=\text{N}$ tautomerism in the dehydropeptide substrate. The same reason probably applies to the lower susceptibility of such a substrate as glycyldehydrophenylalanine to the action of dehydropeptidase I.

The substrates for the dehydropeptidases are distinguished by their possession of an α,β -carbon double bond which is in tautomeric

equilibrium with an adjacent $C=N$ bond in the susceptible linkage. Such substrates may arise in any one of a number of different ways: (a) by the α,β dehydrogenation of saturated peptides, (b) by the desulfuration of cystine peptides, (c) by the condensation between glutamine or asparagine at the amide bond with the carbonyl group of pyruvic acid, and (d) by the splitting of diacylaminopropionic acids.

Whether the action of the dehydropeptidases is reversible is debatable. The splitting of the dehydropeptides leads to the immediate formation of α -aminoacrylic acid or α -aminophenylacrylic acid, etc., which then spontaneously hydrolyze to ammonia and the corresponding α -keto acid. Since a spontaneous reaction is theoretically irreversible, it may be wondered whether the action of an enzyme which breaks down the dehydropeptides can lead to the reverse process or synthesis of dehydropeptides. The presence of the α,β double bond in the dehydropeptides provides the opportunity of adding various side chains at this bond, thus not only saturating this bond but establishing the identity of the amino acid so treated. Thus, hydrocarbon chains, chains with aromatic substituents, or chains with hydroxyl or mercaptan substituents may be conceived as saturating the double bond of the dehydropeptides and forming such natural amino acid residues as leucine, tyrosine, serine, cysteine, etc. Thus, if one or more α,β dehydrogenations occur at various points along an intact polypeptide chain in a protein, followed by substitution at these points by new side chains, a new type of protein can be considered as arising on the polypeptide skeleton of the old. The action of the dehydropeptidases may be viewed as holding this synthetic possibility in check by splitting such unsaturated peptides as fast as they are formed, and thus participating in the anabolic-catabolic equilibria of proteins in tissues. It is conceivable that the unsaturated imino acids so formed may, as in the case of the dehydropeptides themselves, in the presence of suitable systems, accept hydrogen from various donors, and be converted thereby to the corresponding saturated amino acids or peptides.

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ANTIFATTY-LIVER FACTOR OF THE PANCREAS—Present Status

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I. Introduction

Although the significance of the fatty liver did not escape the attention of early investigators such as Virchow and Rosenfeld, our present understanding of the factors that control fat deposition in the liver stems from an observation reported in 1924. In that year it was announced that the surviving depancreatized dog suffers from a massive infiltration of fat in its liver (1,27), and it was in the pursuit of this finding that Best and associates were led to the discovery in 1932 of the lipotropic action of choline (7). The next advance was made by Tudor and Eckstein, when they demonstrated that methionine is also lipotropically active (51). The discovery of these two lipotropic factors, together with the subsequent elucidation of their biochemical interrelation by du Vigneaud and associates (20), gave us a new concept in nutrition, namely that fatty livers result from a deficiency of labile-methyl groups in the diet.

It has been known since 1924 that the development of fatty livers in insulin-treated depancreatized dogs can be prevented by the administration of raw pancreas. When it was recognized that the activity of pancreas fractions upon the liver of this animal could not be accounted for by their choline and methionine contents, another type of antifatty liver factor was therefore brought to light. This unidentified antifatty liver factor contained in pancreas is the subject of the present review.

II. Survival of the Insulin-Treated Depancreatized Dog

Studies on the survival of the depancreatized dog whose diabetes was controlled by the daily administration of insulin were begun soon after the discovery of the antidiabetic hormone. By the addition of raw pancreas to the animals' diets Macleod and associates kept two dogs alive for as long as four years (39), whereas Hedon (30), by somewhat similar treatment, kept a single dog alive for five years. The possibility of the long survival of depancreatized dogs fed diets containing no raw pancreas was questioned, however. Thus the dogs of Fisher (27) and of Allan *et al.* (1) did not live longer than eight months. But their failure to feed diets high in proteins and vitamins to counteract the impaired digestion resulting from loss of pancreatic enzymes probably explains the short survival period reported by these early investigators. A diet meeting the minimum

requirements for a normal dog may be deficient for an animal suffering from impaired absorption.

Survival for more than four years of insulin-treated depancreatized dogs which had been fed a diet adequate in all respects but containing no raw pancreas has been observed in the Berkeley laboratories (13). Because of the difficulties reported by some workers, the treatment of the dogs that survived for these long periods are given here.

TABLE I

MAINTENANCE OF COMPLETELY DEPANCREATIZED* DOGS FOR PERIODS LONGER THAN ONE YEAR WITH DIETS CONTAINING NEITHER RAW PANCREAS NOR FREE-CHOLINE SUPPLEMENTS†

Dog	Weight, kg.		Period of maintenance after pancreatotomy, years	Remarks
	Preoperative	Final		
DA ♀	9.5	7.5	5.5	} Sacrificed while in good condition.
DC ♀	11.9	10.7	5.1	
DB ♂	7.3	7.0	4.2	
G8 ♀	9.0	7.1	3.3	
DF ♀	10.1	9.4	3.1	
DD ♂	8.3	7.4	2.8	Met with accidental death. In good condition at time of accident.
K ♂	11.0	6.5	2.7	Terminated while in good condition.
				Died. Bronchopneumonia and acute pyelitis found at autopsy. Refused food for several weeks before death.
DE ♀	8.1	6.6	2.3	Terminated while in good condition.
G1 ♀	8.0	6.7	1.8	Sacrificed while in good condition.
G3 ♀	8.0	4.5	1.8	Died. Emaciated at time of death despite vigorous appetite throughout.†
G2 ♀	10.4	8.0	1.7	Died. Infected hydronephrosis with multiple small abscesses in right kidney found at autopsy.
DJ ♀	8.9	7.2	1.6	Died. Extensive retroperitoneal hemorrhage and cellulitis with involvement of heart found at autopsy.
A3 ♀	9.3	8.8	1.6	Sacrificed while in good condition.
DG ♀	8.3	6.7	1.6	Died. Acute urinary tract infection found at autopsy. Refused food for two weeks prior to death.
MF ♀	9.6	8.5	1.3	} Sacrificed while in good condition.
A1 ♀	8.8	7.3	1.3	

* Completeness of pancreatectomy was confirmed in all cases at necropsy.

† It should be noted that the diets employed were not choline-free. The term "supplements" refers to choline in addition to that already present in the high-calorie, high-protein, high-vitamin diet fed in this laboratory.

‡ The degree of emaciation shown by this dog was not observed in other animals. Such variations might be the result of variations in the degree of absorption to be found after pancreatectomy (see page 180).

For several weeks before pancreatectomy they were fed a diet high in calories, proteins, and vitamins. Following pancreatectomy each dog was injected with 8 units of insulin twice daily, at 8 A.M. and 4 P.M. Just before each injection, they were fed the following mixture: 280 g. raw lean beef, 50 g. sucrose, and 7 g. bone ash. Vitamin supple-



Fig. 1. Dog DA, 5.5 years after pancreatectomy (15).



Fig. 2. Dog DC, 5.1 years after pancreatectomy (15).



Fig. 3. Dog DB, 4.2 years after pancreatectomy (15).

ments in the form of cod liver oil and a rice bran concentrate were added to the diet mixture twice each week (15). A record of the dogs maintained under the above conditions from one to five and one-half years is shown in Table I. Dogs DA, DB, and DC are specially significant because their survival periods are the longest so far recorded for completely depancreatized dogs maintained on a diet containing

no pancreas. Their appearance at the time they were sacrificed is shown in Figures 1-3.

III. Pathological Changes Appearing in Insulin-Treated Depancreatized Dog Fed a Diet of Lean Meat, Sucrose, Salts, and Vitamins

Despite their long survival, these dogs are far from normal. The faulty absorption imposed by the loss of pancreatic juice probably accounts for their inability to gain weight and for the loss of weight observed in many of them. In addition, the following defects have been observed: (1) a lipide disturbance characterized by a fatty liver and a decreased concentration of blood lipides, (2) cirrhosis of the liver, and (3) cataracts.

A. THE FATTY LIVER

The deposition of excessive amounts of fat in the liver of the insulin-treated depancreatized dog, first recognized by Allan *et al.* (1), has been amply confirmed. A cardinal feature of this fatty liver is that it cannot be accounted for by failure of this dog to ingest adequate amounts of proteins, vitamins, salts, and calories. It appears in the depancreatized dog fed amounts of these dietary constituents that are more than sufficient for maintaining the *intact* dog normal in all respects.

The livers of normal and insulin-treated depancreatized dogs fed the same diet are compared in Table II. The livers of the depancreatized dogs, in addition to containing large amounts of fat, are friable and enlarged. Normal dogs that weighed 13-14 kg. contained 250-280 g. of liver, approximately 2% of the animal's body weight. Insulin-treated depancreatized dogs weighing from 7-13 kg., on the other hand, contained from 500-1100 g. of hepatic tissue, which amounted to about 6-9% of their final body weight. Even when related to the preoperative body weight, the ratio of liver weight to body weight in the depancreatized dog is still greater than normal.

Total lipides may rise as high as 48% of the wet weight of the liver in the depancreatized dog (33), approximately twelve times the maximum amount found in normal dogs fed a similar diet. Since, however, a marked increase also occurred in the size of the livers of the depancreatized dogs, the actual gain in liver lipides can best be gaged by comparing the absolute amounts found in both types of dogs. The

TABLE II
COMPARISON OF LIVER LIPIDES* OF NORMAL AND INSULIN-TREATED DEPANCREATIZED DOGS

Dog	Weight, kg.		Period on diet, † weeks	Period since pan-creatotomy, weeks	Liver							
	Initial	Final			Weight, g.	Percent of body wt.	Cholesterol, %			Phospho-lipides, %	Total fatty acids, %	Total lipides, %
							Total	Free	Ester			
Normal	14.0	14.3	20		282	2.0	0.21	0.18	0.03	2.22	4.17	4.38
	13.1	13.5	20		255	1.9	0.26	0.21	0.05	3.07	3.82	4.08
	13.7	13.1	20		260	2.0	0.28	0.19	0.09	2.82	2.91	3.19
Depancrea-tized	12.5	12.5	20	20	1100	8.8	0.38	0.18	0.20	2.06	20.4	20.8
	15.0	7.8	20	20	500	6.4	0.51	0.20	0.31	1.98	15.1	15.6
	10.2	7.7	20	20	637	8.3	0.41	0.32	0.09	2.55	20.5	20.9

* All lipid values expressed as percentages of wet tissue.

† The normal dogs received per kilogram per day 30 g. lean meat and 5 g. sucrose. The depancreatized dogs were fed 250 g. meat and 50 g. sucrose twice daily. All diets were supplemented daily with vitamins and salts.

liver of a normal dog weighing 11 kg. contained about 10 g. of total lipides, whereas the liver of a depancreatized dog weighing 7 kg. contained 352 g.

The various lipide constituents do not share equally in the massive deposition of lipides in the liver of the depancreatized dog. The total amount of phospholipides contained in the liver of this dog increases but, because the liver is enlarged, the phospholipide content per gram of tissue is decreased (33). In the *normal* dog, phospholipides account for as much as 60% of the total lipides in the liver, whereas, because of the large increase in neutral fat, phospholipides account for only 2-12% of the total lipides in the depancreatized dog liver. Table III shows that the various types of phospholipides are present in approximately normal proportions in the livers of insulin-treated depancreatized dogs. Choline-containing phospholipides in the fatty livers of depancreatized dogs varied from 43 to 65%, which is the range reported for the normal dog (50).

TABLE III
CHOLINE-CONTAINING PHOSPHOLIPIDES IN LIVERS OF DEPANCREATIZED DOGS

Number of dogs	Total fatty acids, %	Phospholipides	
		Total, %	Choline-containing as per cent of total
14	2.4 to 4.4*	1.85	43-65
7	6.7 to 11.2†	1.56	42-61
6	11.9 to 21.6†	1.05	43-64

* Normal fat content maintained by the feeding of raw pancreas for twenty weeks.

† These dogs received no raw pancreas for twenty weeks.

Phospholipides and cholesterol account for only a small fraction (less than 6%) of the total lipides that accumulate in the livers of depancreatized dogs. As much as 97% of the fatty acids is present as triglycerides.

As judged by iodine numbers, the liver fatty acids are of a higher degree of unsaturation in the normal than in the depancreatized dog. Thus in the normal dog the iodine numbers of liver fatty acids were 131-136, whereas in the depancreatized dog the values were 68-71 (33). But the relatively low degree of unsaturation in the depancreatized dog is to be expected in view of the low proportion of fatty

acids present as phospholipides, the constituent chiefly responsible for the high degree of unsaturation of normal liver fatty acids.

It should be pointed out that chronic undernutrition does not account for the tremendous increase of liver fat in the depancreatized dog. No increase in the concentrations of lipides was found in normal dogs in which losses of 20 to 50% in their body weights had been induced by subjecting them to low caloric intakes for a period of twenty weeks (21).

1. Rate of Fat Deposition in Livers of Insulin-Treated Depancreatized Dogs

The deposition of the excessive amounts of fat in the liver of the insulin-treated dog fed a diet adequate in calories, proteins, salts, and vitamins, but containing no raw pancreas, is not a rapid process. The rate at which fatty livers develop under these experimental conditions was shown in a study of 27 dogs sacrificed at intervals from 3.5 to 36 weeks after pancreatectomy. The data are recorded in Table IV. Although fatty livers were observed as early as 3.5 weeks after excision of the pancreas, the occurrence of such livers was not a constant finding at this time interval. Not only the degree of fat infiltration but also the time of onset of such changes show considerable variation. It required a period of *at least* sixteen weeks to insure a consistent finding of fatty acids in excess of 14% in the livers of these dogs. This variability in the development of fatty liver has important implications in the assay of the antifatty liver factor of the pancreas, which is discussed on page 195.

2. Fat Content of Livers of Depancreatized Dogs That Survived from 4.2 to 5.5 Years

Once the liver has become fatty, this fatty condition persists for a long time; and in a single dog as much as 20% fatty acids was found in the liver three years after pancreatectomy (33). But, if the animals survive long enough, a spontaneous decline in the fat content of the liver may occur. In three dogs that survived for 4.2 to 5.5 years (DA, DB, DC, Table IV), approximately normal concentrations of fatty acids were found, although the total amounts of fatty acids present were still far in excess of the normal because of the fact that the size of the livers failed to regress as fat left them.

TABLE IV

RELATION OF TIME AFTER PANCREATECTOMY TO FATTY-ACID CONTENT OF LIVERS
IN COMPLETELY DEPANCREATIZED DOGS MAINTAINED WITH INSULIN

Dog		Period depancreatized	Liver	
No.	Weight, kg.		Weight, g.	Total fatty acids, %
D100	6.6	3.5 weeks	315	11.0
D106	4.0	3.5	225	31.8
D1*	11.5	4.5	480	18.6
D105	9.5	6.5	525	7.08
DM	9.7	9	700	29.0
D39	6.0	9	280	6.93
D40	6.0	9	400	5.58
D41	6.6	9	355	10.0
D103	7.2	9.5	327	21.6
D34	7.4	10	395	28.7
DN	7.1	10	770	43.2
D33	8.8	11	485	28.2
D44	4.6	11	235	13.6
D35	7.8	11.5	390	14.0
D38	7.3	12	490	6.85
D47	9.8	14.5	375	5.28
D94	4.8	14.5	260	10.6
D45	5.5	15	335	8.35
D58	6.9	15	750	41.4
D59	8.5	19.5	360	28.5
D56	7.0	20	385	21.0
D79	6.0	20	290	25.2
D84	9.0	20.5	650	29.2
D52	7.0	21		14.4
DH	9.6	25	690	36.1
DK	7.0	34	565	26.3
D85†	9.1	36	540	27.2
DB*	7.0	4.2 years	400	2.46
DC	10.7	5.1	480	2.71
DA	7.5	5.5	565	4.90

* Male. All other dogs were females.

† Received double portions of meat for last sixteen weeks.

TABLE V

PLASMA LIPIDES IN THE INSULIN-TREATED DEPANCREATIZED DOG*

Period after pancre- atectomy, weeks	Body weight, kg.	Cholesterol			Total fatty acids	Phospho- lipides	Total lipides
		Total	Free	Ester			
Before	13.2	225	57	168	600	383	825
4	11.0	119	46	73	351	230	470
10	10.3	107	52	55	419	235	526

* Dog D374. All lipid values expressed as milligrams per 100 ml. plasma.

B. PLASMA LIPIDES

Significant changes also occur in plasma lipides of the insulin-treated depancreatized dog (Table V). Their concentration decreases while fatty livers are developing, but a pronounced fall in the former may appear long before a measurable increase in the fat content of the liver has occurred. A drop of 30–50% below normal in the level of plasma lipides has been observed as early as two to three weeks after pancreatectomy. Following this initial rapid drop, the concentration of plasma lipides may either show no further change or decrease slowly to lower levels during the next ten to twenty weeks.

All lipid constituents of the plasma are decreased by pancreatectomy, but the most pronounced drop is usually found in esterified cholesterol. Free cholesterol, on the other hand, shows less change than any other lipid constituent. Marked drops are noted in both total fatty acid and phospholipid levels. It is of interest that the plasma phospholipides in the depancreatized dog, as in the normal dog, are all of the choline-containing type (21). It is not known whether the relative proportions of lecithins and sphingomyelins in the plasma of the insulin-treated depancreatized dogs are altered.

C. ABSORPTION OF PROTEINS, FATS, AND CARBOHYDRATES

The dog deprived of pancreatic enzymes either by complete excision of its pancreas or by ligation of its excurrent ducts would be expected to suffer from impaired absorption of both proteins and fats. Surprisingly enough, however, the extent to which their absorption is interfered with by these procedures varies considerably from dog to

TABLE VI

ABSORPTION OF FAT AND PROTEIN BY DOGS DEPRIVED OF PANCREATIC JUICE

Investigators (reference number)	Method by which pancreatic juice was excluded	Fat absorbed, %	Nitrogen absorbed, %
Pratt, 1907 (28)	Duct ligation	4.8 to 76.6	22.2 to 61.7
Vinsentini, 1914 (53)	Duct ligation	28.7 to 44.0	
Vinsentini, 1914 (53)	Pancreatectomy	8.7 to 25.7	
Cruikshank, 1915 (17)	Pancreatectomy	32.6	78
Pratt <i>et al.</i> , 1931 (46)	Duct ligation	93.6	85.3
Pratt <i>et al.</i> , 1931 (46)	Pancreatectomy	95.2	56.2
Handelsman <i>et al.</i> , 1934 (29)	Duct ligation	41.5 to 93.7	47.0 to 60
Selle, 1931 (48)	Pancreatectomy	av. 89.5	33–73
Ralli <i>et al.</i> , 1938 (47)	Duct ligation	47 to 72	
Entenman <i>et al.</i> , 1946 (21)	Pancreatectomy	29 to 95	26–65

dog. This is brought out in Table VI, in which are summarized the significant observations made during the past forty years. From 20 to 70% of ingested nitrogen remains unabsorbed in the intestine of the dog deprived of pancreatic juice. No explanation can be offered for this variability, and still less is known of the degree to which the unabsorbed protein is hydrolyzed. That there is an actual failure in protein digestion is apparent from the condition of the feces in depancreatized dogs which contain large amounts of cell nuclei and striated muscle fibers. There can be little doubt that failure in digestion and absorption accounts for the inability of the insulin-treated depancreatized dog to maintain its body weight when fed diets containing no raw pancreas.

Table VI also shows the degree to which fat absorption is impaired in dogs deprived of pancreatic juice. Ingested fat does not pass through the intestinal tract unchanged, however, for as much as 70% of the fecal fat is in the form of fatty acids and soaps. The reason for the occurrence of splitting in the absence of pancreatic juice is not clear. Some believe that the lower pH in the duodenum of the depancreatized dog permits gastric lipase to act there; others ascribe splitting to the lipase of the succus entericus; and still others state that the splitting is due to bacterial action (28). If splitting had occurred in the duodenum, it seems reasonable to believe that the absorption of fat would not have been impaired.

In contrast to fats and proteins, carbohydrates seem to be well absorbed by the dog lacking pancreatic juice (28).

D. BODY WEIGHT

The completely depancreatized dog, in spite of the administration of insulin and a diet not only high in calories but containing more than adequate amounts of protein, salts, and vitamins, loses weight. This weight loss, however, was not found to be uniform among the dogs studied in the Berkeley laboratories, and probably reflects the variability in the degree of impaired absorption that results from pancreatectomy. A dog may, in twenty weeks, lose 50% of its pre-operative weight or may show no loss whatever, but the average weight loss in this period does not exceed 30%. The most pronounced weight loss occurs, as a rule, during the first three to four weeks after pancreatectomy.

E. CATARACTS

A high incidence of cataracts has been observed in insulin-treated depancreatized dogs that have survived for some time (13). Bilateral cataracts appear as early as one year after pancreatectomy. The changes vary in degree from faint striations to diffuse irregular opacification. All cataracts were either incipient or immature. As judged by ophthalmoscopic examination, the lenticular opacities were similar to those found in early senile cataracts of human subjects.

F. CIRRHOSIS OF THE LIVER

An abnormal degree of fibrosis and cirrhosis has been frequently observed in the livers of insulin-treated depancreatized dogs (10). Extensive interlobular fibrosis associated with hyaline or colloid degeneration of many cells was found in the livers of dogs that survived for periods longer than one and one-half years. This fibrous tissue proliferation frequently gave a prominence to the portal spaces not seen in the normal dog. By the time these dogs had survived for four years or more, this process was so pronounced that the picture of a well-advanced cirrhosis of the liver was present (Fig. 4). Such livers presented the characteristic hobnailed appearance, their surfaces being covered by nodules of varying size, the largest of which measured 10 mm. in diameter. Infection and obstruction of the extrahepatic bile passages appeared to be ruled out as causative agents in the production of this liver scarring. The long-continued presence of large amounts of fat appeared to be a predisposing factor.

IV. Effect of Raw Pancreas on Lipide Metabolism of the Insulin-Treated Depancreatized Dog

Macleod and associates were the first to observe that the feeding of raw pancreas improved the condition of the feces and the general well-being of the insulin-treated depancreatized dog (38,39). Raw pancreas was fed for the first time in the Toronto laboratories during November, 1923, and in 1930 Macleod (39) presented the clinical histories of two depancreatized dogs kept alive for about five years by treatment with insulin and a diet containing 50 g. of raw pancreas daily in addition to lean meat and sucrose. It is of interest to learn what prompted these early workers to introduce the raw glandular tissue into the diet. The finding of imperfect digestion of food in the insulin-treated depancreatized dog in association with fatty changes

in their livers led Macleod (39) to state in 1930: "Believing that the absence of pancreatic ferments, upon which this inadequate digestion obviously depended, might be related to the hepatic changes, it was decided to add raw pancreas to the daily diet." It should be pointed out, however, that this statement was made by Macleod before the lipotropic effects of choline or protein were discovered.

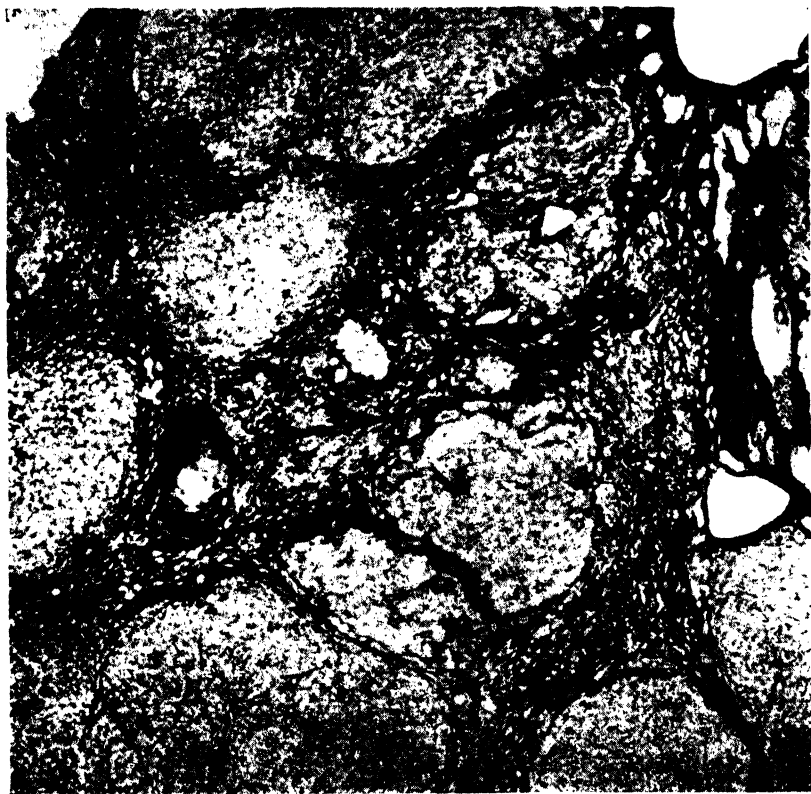


Fig. 4. Well-advanced cirrhosis in the liver of dog DA found 5.5 years after pancreatectomy (10).

A. LIVER LIPIDES

The effect of raw pancreas in *preventing* the infiltration of fat in the liver is shown in Table VII. It is apparent that the daily ingestion of 250 g. of raw pancreas (in addition to the regular diet) was effective in preventing the abnormal deposition of fat in the liver for periods

TABLE VII
PREVENTION OF FATTY LIVERS IN INSULIN-TREATED DEPANCREATIZED DOGS BY
INGESTION OF RAW PANCREAS

Dog	Body weight, kg.		Period receiving raw pancreas,* weeks	Liver	
	Preoperative	Final		Weight, g.	Total fatty acids, %
D1	13.9	13.5	4	433	3.1
D5	10.4	10.7	10	500	2.7
D99	8.5	9.0	57	440	3.2

* The feeding of 250 g. raw pancreas daily was begun immediately after pancreatectomy.

extending from four weeks to one year. The ingestion of raw pancreas is also effective in maintaining body weight and nutritive state of the dogs.

That the ingestion of raw pancreas can remove the accumulated fat in the liver is illustrated in Table VIII; in the dogs referred to the

TABLE VIII¹
CURATIVE ACTION OF RAW PANCREAS ON FATTY LIVERS OF INSULIN-TREATED
DEPANCREATIZED DOGS*

Dog	Weight, kg.	Period of observation		Liver	
		No pancreas in diet, weeks	Pancreas added to diet, weeks	Weight, g.	Total fatty acids, %
D20	8.7	First 16	Last 4	490	29.2
D25	10.8	First 14	Last 5	850	23.1
D24	9.1	First 33	Last 6	800	26.7
D12	8.5	First 29	Last 6	420	11.3
D61	9.5	First 18	Last 15	490	8.6
D66	7.9	First 20	Last 16	500	11.5

* Each dog received daily 250 g. raw pancreas in addition to the lean-meat diet.

feeding of raw pancreas was begun after fatty livers were already present. A striking feature of its curative effect is its slow action. While the ingestion of 250 g. of the raw glandular tissue each day following pancreatectomy (*i.e.*, from a time when the fat content of the liver was still normal) completely inhibited the deposition of abnormal amounts of fat, the administration of the same amount of raw pancreas for as long as 16 weeks, begun at a time when fatty livers were present, failed to empty the liver completely of its abnormal

amounts of fat. A feeding period as short as four or five weeks apparently had no appreciable effect on fatty livers. Once the liver is allowed to become fatty, therefore, raw pancreas must be fed for a period longer than 16 weeks if the fat content of the liver is to be restored to normal.

B. BLOOD LIPIDES

The decrease in the lipid content of the blood, which is characteristic of the depancreatized dog receiving a lean-meat and sucrose diet, can be prevented or corrected by the addition of raw pancreas to the diet. If immediately following pancreatectomy raw pancreas is added to the diet, the blood lipides remain at or above the preoperative

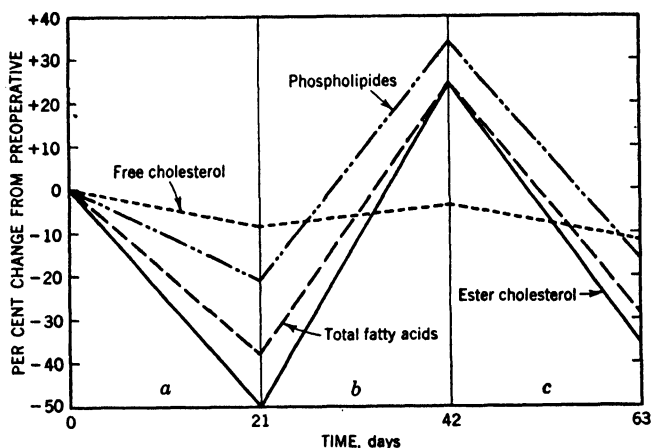


Fig. 5. The blood lipid changes produced in an insulin-treated depancreatized dog by the addition and omission of raw pancreas from the diet. During period A the dog received the lean-meat and sucrose diet. During period B, 250 g. raw pancreas was added daily to this lean-meat and sucrose diet. During period C the raw pancreas was omitted from the diet.

levels. On the other hand, if no pancreas is fed the lipid content of blood drops markedly and reaches a plateau in three to four weeks. The addition of pancreas to the diets at a time when the lipid levels are low produces an immediate increase in the lipid content of the blood, and within three weeks the blood lipid content is again normal or above normal.

Even though all constituents respond to pancreas therapy, they do not respond to the same extent (Fig. 5). The most marked rise usually occurs in the esterified cholesterol fraction, but notable increases are also observed in the levels of total fatty acids and phospholipides. As a rule, little change occurs in the levels of free cholesterol.

The amount of pancreas necessary to keep the blood lipides of the depancreatized dog at preoperative levels is in fact very small. Dogs were fed the equivalent of 5.5 g. pancreas per day. It is clear from Table IX that this amount of pancreas is as effective in keeping the blood lipides at normal levels as 250 g. (Fig. 5).

TABLE IX
MAINTENANCE OF NORMAL BLOOD LIPIDE LEVELS IN THE DEPANCREATIZED DOG
BY INGESTION OF PANCREAS*

Period after pancrea- tectomy, weeks	Period receiving pancreas fraction AR, † weeks	Amount AR per day, g.	Cholesterol			Total fatty acids	Phos- pho- lipides	Total lipide
			Total	Free	Ester			
Before			168	135	33	427	420	595
20	20	1	198	157	41	454	471	652

* Dog D77. All lipide values expressed as milligrams per 100 ml. whole blood.

† 1 g. AR was derived from 5.5 g. raw pancreas. It is the residue that remained after extraction of raw pancreas with acetone and ether.

The rise in blood lipides (Fig. 5) does not result from the *extra calories* contained in the ingested glandular tissue, for a similar response is not observed when this tissue is fed to normal dogs. Moreover, the observation that in the depancreatized dog 5 g. raw pancreas is just as effective as 250 g. would also appear to rule out presence of the extra calories as an explanation for the rise in blood lipides.

V. Relation of External Secretion of the Pancreas to Fat Metabolism

Macleod, in 1926, presented two possible explanations for the fatty livers of the insulin-treated depancreatized dog: either that the digestive disturbance produces amines or similar toxic substances that are carried by the portal blood to the liver, or that the pancreas, quite apart from its function in producing insulin, also secretes some other hormone which is necessary for the physiologic integrity of the liver cell (38). A third possibility was recognized by this worker when he

stated four years later that the absence of pancreatic ferments might be related to the hepatic changes (39). No experimental support, however, was offered by Macleod for any of these three views.

Since the loss of the external secretion of the pancreas is the most obvious defect in the insulin-treated depancreatized dog, its relation to the development of fatty livers will be considered first. Apart from pancreatectomy, pancreatic juice can be excluded from the intestinal tracts by fistula or by ligation of the excurrent ducts of the gland. The fistula procedure would appear to be the method of choice; for, theoretically at any rate, it offers a means of completely ridding the body of the external secretion of the pancreas without interfering with other possible functions of the gland. Unfortunately, dogs furnished with pancreatic fistulas that allow *none* of their pancreatic juice to be absorbed from the intestinal tract cannot be maintained in good condition for long periods. Ligation of pancreatic ducts, though not without difficulties, has been used in the study of hepatic dysfunction. The first to produce fatty livers in dogs by this procedure were Aubertin *et al.* (4). Their findings were confirmed by Ralli *et al.* (47) and by Person and Glenn (44). But in 1936 Dragstedt and co-workers failed to observe fatty livers in dogs in which the pancreatic ducts had been ligated (52). A reinvestigation of this subject was therefore undertaken by Montgomery and associates.

A. LIPIDE CHANGES IN THE DOG SUBJECTED TO LIGATION OF PANCREATIC DUCTS

The method used by Montgomery *et al.* in the Berkeley laboratories for ligation of pancreatic ducts was designed to insure complete severance of *all* communications between pancreas and duodenum and to prevent reestablishment of these communications by duct recanalization (40). Despite elaborate precautions, however, recanalization may occur; and only by careful *post mortem* inspection and dissection in the region of the pancreas can it be ascertained whether all connections between pancreas and duodenum have remained occluded during the periods of observation. In all dogs referred to below as duct-ligated, complete separation between the pancreas and duodenum was so established.

Soon after ligation of all ducts, the characteristics of the acinous tissue are lost and the gland becomes greatly atrophied. The residual structure is rarely thicker than 2 mm.

TABLE X
LIVER LIPIDES OF DUCT-LIGATED DOGS FOLLOWING PANCREAS INGESTION

Treatment		Dog	Weeks after duct ligation	Body weight, kg.		Liver			
Insulin injected	Raw pancreas in diet			Pre-operative	Final	Wt., g.	Total cholesterol, %	Phospholipides, %	Total fatty acids, %
None	None	E7	24	11.6	8.0	360	0.41	1.75	11.4
		E12	21	9.6	8.9	370	0.66	1.41	29.0
		E20	12	12.0	6.6	510	0.32	1.93	35.5
4 units twice daily	None	E21	20	5.0	6.7	255	0.28	1.60	14.7
		E22	20	5.0	5.6	275	0.39	1.69	20.4
		E25	20	9.0	7.6	185	0.32	1.98	6.32
None	250 g. daily	E15	20	17.7	18.9	345	0.24	1.82	1.87
		E16	20	9.0	8.9	220	0.25	2.18	2.28
		E17	20	7.8	8.9	220	0.26	2.71	3.10

Following exclusion of pancreatic juice from its intestinal tract, the dog loses weight even when its caloric and protein intakes are far in excess of the normal. Dogs in which the loss in weight was particularly marked suffered from muscular weakness. The injection of 4 units of insulin twice daily prevented the loss of weight in the duct-ligated dog. This was somewhat surprising since in duct-ligated dogs that received no insulin the blood sugar levels were within the normal range.

Montgomery *et al.* (40) found that complete exclusion of pancreatic juice from the intestine induced fatty livers (Table X). The earliest observation was made twelve weeks after all communications between pancreas and duodenum had been successfully disconnected, and by this time as much as 35% of fatty acids had accumulated in the liver. Popper and Necheles also reported finding fatty livers in two dogs twelve months after all pancreatic ducts had been occluded (45).

The accumulation of lipides in the liver of the duct-ligated dog is a slow and variable process. The fatty liver is the result of neutral-fat infiltration. The concentration of esterified cholesterol also increases, but free cholesterol and phospholipides show little change. In all of these respects the duct-ligated dog resembles the insulin-treated depancreatized dog.

The administration of insulin corrected the weight loss in the duct-ligated dogs, as noted above, but failed to prevent the accumulation of excessive amounts of fat in their livers. These fatty changes were prevented, however, when the administration of raw pancreas was begun immediately after duct ligation. Duct-ligated dogs fed raw pancreas showed no weight loss even when they received no insulin (40).

Complete occlusion of pancreatic ducts in dogs also leads to a decrease in blood lipid constituents (23). Insulin failed to prevent this decrease. The ingestion of pancreas begun immediately after duct ligation prevented the decline in the level of the blood lipides. The addition of the glandular tissue to the diet at a time when low lipid levels had already been established restored their levels to normal.

The similarity of the action of pancreas in the duct-ligated and in the insulin-treated depancreatized dog suggests that the mechanism whereby fatty livers are produced in both types of animal preparation is the same.

B. LIPIDE-METABOLISM-STIMULATING PROPERTIES OF PANCREATIC JUICE

Although the observations thus far presented are not incompatible with the view that a factor or factors controlling lipide metabolism resides in the external secretion of the pancreas, it is nevertheless necessary to point out that loss of an internal secretion of the pancreas—other than insulin, of course—resulting from atrophy of the gland induced by duct ligation could also explain these observations. Strong evidence, however, favoring pancreatic juice as the controlling medium is provided by the finding that abnormal changes in liver and blood lipides in insulin-treated depancreatized dogs and in duct-ligated dogs can be prevented by introducing pancreatic juice into their diets (24,43).

Clear, practically odorless pancreatic juice was obtained from dogs by means of a modified Elman-McCaughan fistula (43). Juice-producing animals were fed a diet of lean meat and sucrose supplemented with vitamin concentrates and salts. In addition, each dog received daily 800–1000 ml. of Ringer's solution intravenously.

The efficacy of orally administered pancreatic juice in preventing the development of fatty livers in depancreatized dog is shown in Table XI. The control dog received the lean-meat and sucrose diet but no pancreatic juice. Twenty weeks after pancreatectomy its liver contained 22.5% fatty acids. The other dogs during the several months of observation received pancreatic juice in their diets in amounts varying from 10 to 200 ml. per day. The livers of all dogs examined had normal fat contents; and, as can be seen from the Table XI, 10 ml. pancreatic juice daily (*i.e.*, 5 ml. with each meal)

TABLE XI
PREVENTION OF FATTY LIVERS IN INSULIN-TREATED DEPANCREATIZED DOGS
BY FEEDING PANCREATIC JUICE

Dog	Weight, kg.		Pancreatic juice		Liver	
	When pancreatic juice feeding started	Final	Period fed, weeks	Amount per day, ml.	Weight, g.	Total fatty acids, %
D1	Control	7.2	None	None	493	22.5
D6	14.2	10.6	20	200	590	2.5
D9	8.8	9.1	20	100	624	2.6
D12	16.9	14.7	20	50	556	3.0
D15	7.2	8.5	20	20	360	4.2
D20	13.4	8.9	22	10	615	3.0

was just as effective as 200 ml. in inhibiting the infiltration of fat in the liver. Pancreatic juice also protected the duct-ligated dog from fatty infiltration of the liver (43). Pancreatic juice resembles raw pancreas in its action upon blood cholesterol, phospholipides, and total fatty acids (24).

The results presented in this section leave no doubt that the abnormal changes in lipide metabolism produced either by excision of the pancreas or by duct ligation can be corrected by the daily ingestion of pancreatic juice. These observations establish an essential function for the external secretion of the pancreas, namely the maintenance of normal lipide levels in liver and blood. It would appear that the release of pancreatic juice into the intestinal tract is necessary for inhibiting excessive amounts of fat from being deposited in the liver.

VI. Relation of Choline to the Antifatty-Liver Factors Contained in Pancreas

The beneficial effect of crude egg yolk lecithin upon the liver of the insulin-treated depancreatized dog was first described by Hershey in 1930 (31). Normal livers were found by this observer in two such animals sacrificed after one year of lecithin feeding. These findings were confirmed and extended in later reports by Hershey and Soskin (32) and by Best and Hershey (6). The active component of the lecithin molecule was soon identified as choline by Best and associates (7). The action of choline on fatty livers of the insulin-treated depancreatized dog was confirmed by Kaplan and Chaikoff (35), who showed, however, that, while the daily administration of choline begun immediately after pancreatectomy readily prevented the deposition of abnormal amounts of fat in the liver, the *curative* action of choline (by which is meant its action upon livers in which large amounts of fat have been deposited before the feeding of choline was initiated) was a slow one. In this respect choline resembles raw pancreas (34). Choline is also an effective agent for the prevention of fatty livers in the duct-ligated dog (26). This additional action of choline provides further evidence of a similarity in the mechanism by which fatty livers occur in the two types of experimentally produced conditions.

The investigations just described led Best and associates to regard the choline content of pancreas as accountable in part for the antifatty liver action of this tissue in the insulin-treated depancreatized

dog. When later it was shown that betaine and protein exert lipotropic effects, they were careful to suggest that some of the activity of pancreas might be due to these two compounds or closely related factors (9). This explanation of the antifatty-liver action of pancreas was questioned, however, for it did not explain the failure of large amounts of lean meat (39) and of liver (52), both of which are about as rich as pancreas in methionine and choline, to prevent the appearance of fatty livers in the insulin-treated depancreatized dog.

An answer to the relation of choline to the antifatty-liver factor of pancreas was sought by comparing the minimum effective dose of choline with the minimum amount of pancreas necessary for maintenance of normal livers in insulin-treated depancreatized dogs (22). It was shown that 35 mg. choline per kg. per day are sufficient to inhibit completely the deposition of abnormal amounts of fat in the livers of insulin-treated depancreatized dogs. This amount of choline kept the fatty acid content of livers at 3 to 4% levels even when the period of observation was extended to five months. Although this may not be the smallest effective dose of choline, it was found that a daily dose of 15 mg. per kg. per day was not sufficient.

Supplementing the diet of the insulin-treated depancreatized dog daily with 1 g. of a dried defatted preparation of pancreas designated AR, (the residue that remains after extraction of pancreas with acetone and ether, 22) which was derived from 5.5 g. of original glandular tissue, was found sufficient to prevent completely the development of fatty livers. Since this gram of AR contains less than 15 mg. choline, it is apparent that the antifatty liver property of this fraction cannot be accounted for by its choline content. Further evidence for this view is provided by the observation that the antifatty-liver substance in AR is completely destroyed by heat, whereas similar treatment does not destroy choline. The daily administration of steam-treated AR in amounts equal to four times the dose found effective for the unheated fraction failed to inhibit fatty livers.

It was pointed out on page 190 that the daily ingestion of as little as 10 ml. pancreatic juice is sufficient to prevent fatty livers. Since 100 ml. fresh pancreatic juice contained less than 1 mg. choline (42), it is also evident that the antifatty-liver effects of pancreatic juice cannot be accounted for by its choline content.

It should be stressed now that, while the above considerations definitely establish that pancreas and pancreatic juice contain a noncho-

line antifatty-liver factor, they do not rule out the possibility that additional amounts of choline are made available to the insulin-treated depancreatized dog by the presence of pancreas fractions or juice in the gastrointestinal tract.

VII. Preparation of Pancreas Fractions Rich in Antifatty-Liver Factor

Investigations aimed at the isolation of the unidentified factor in the pancreas were undertaken first in the Chicago laboratories of Dragstedt and associates (18) and later in the Berkeley laboratories by Entenman *et al.* (25).

A. LIPOCAIC

In 1936 Dragstedt *et al.* obtained an active fraction by alcoholic extraction. According to their report of that year it was prepared as follows: Raw pancreas was extracted four times with 95% alcohol; the extracts were concentrated at room temperature to a brown paste, and the latter was washed repeatedly with ethyl ether. The alcohol-soluble, ether-insoluble residue was designated lipocaic. According to these workers, the feeding of 2–4 g. per day of it cured fatty livers in insulin-treated depancreatized dogs; these amounts of lipocaic were derived from about 100 g. raw pancreas. In a subsequent report (19), the Chicago workers, by modifying the method for isolating lipocaic, reduced the effective dose to 0.75 to 1.5 g. per day. Lipocaic has the following properties: It is soluble in water, 5% saline, 60% ethanol, and glacial acetic acid, and insoluble in 70% ethanol. It is precipitated in the presence of 0.75-saturated ammonium sulfate and from its solution in glacial acetic acid by 3–4 volumes of ethyl ether. It contains no proteolytic activity and is not dialyzable.

Dragstedt and associates claimed to have demonstrated that a purified preparation of lipocaic (obtained by precipitation of the crude material with 0.75-saturated ammonium sulfate and fractionation of the precipitate with alcohol) was active in preventing fatty livers in insulin-treated depancreatized dogs when administered *subcutaneously* in doses of 55 mg. per kg. (16). A detailed report of this work has not yet appeared, and consequently judgment as to its significance must be withheld for the present. If confirmed, however, it would provide evidence for the concept that lipocaic is an internal secretion.

B. ANTIFATTY-LIVER FRACTIONS PREPARED FROM PANCREAS
BY ENTENMAN AND ASSOCIATES

In 1944 Entenman *et al.* reported various procedures for the preparation of pancreatic fractions rich in the antifatty-liver factor (25). The extraction procedures used depended upon the observation that the factor is soluble in dilute acid, is precipitated by ammonium sulfate between 0.25 and 0.50 saturation, and is nondialyzable. The oral administration of 60 mg. of fraction 27C, the most active fraction reported up to that time, was sufficient to prevent the development of fatty livers in insulin-treated depancreatized dogs weighing from 11.5 to 16.2 kg. It is of interest that, while preventing the development of fatty livers, this amount of 27C failed to maintain the dog's weight. During an examination of the properties of the moiety insoluble in 0.5-saturated ammonium sulfate, it was observed that an active fraction could be precipitated from its aqueous solution at pH 4 by means of 60% alcohol. This finding was somewhat surprising since Dragstedt's lipocaic is soluble in the presence of this concentration of alcohol.

The scheme employed recently in the Berkeley laboratories for isolation of various active fractions involved alcoholic fractionation of a dilute-acid extract of pancreas. Some characteristics of two highly active fractions are recorded in Table XII. Both are of interest, for they bring to light the potency of the antifatty-liver factor contained in pancreas; the daily feeding of 6–22 mg. completely prevented fatty livers in insulin-treated depancreatized dogs weighing 10–15 kg.

TABLE XII

SOME PROPERTIES OF PANCREAS FRACTIONS HIGHLY ACTIVE IN PREVENTION OF
FATTY LIVERS IN INSULIN-TREATED DEPANCREATIZED DOGS

Fraction	Amount found to prevent fatty livers, mg.	Solubility in 60% ethanol	Proteolytic activity*
63A	22	Soluble	None
62C	6	Insoluble	None

* Proteolytic activity measured by method of Anson (3).

Entenman and Chaikoff have tested the antifatty-liver activity of pancreas fraction prepared by Barnes, Bosshardt, and Ciereszko of Sharp and Dohme Laboratories. A residue obtained from raw pancreas by treatment with acid alcohol was used as starting material;

a water-soluble, acetone-insoluble fraction prepared from this residue was found to be rich in antifatty-liver activity. The oral administration of 10 mg. per day of the fraction was sufficient to prevent fatty livers in insulin-treated depancreatized dogs. It contained no proteolytic activity, as judged by *in vitro* tests (3).

C. METHOD OF ASSAY FOR ANTIFATTY-LIVER ACTIVITY OF PANCREAS FRACTIONS

In testing for the presence of lipocai in their fractions, Dragstedt and associates employed the following criteria in depancreatized dogs: curative action upon fatty livers as checked by biopsy, improvement in glucose excretion and insulin tolerance, relief of hypoglycemia, and improvement of the nutritive state (2,19). The test period occupied four to six weeks.

It was observed in the Berkeley laboratories, however, that the curative action of either raw pancreas or choline is slow and irregular (34,35) and that a single biopsy sample of the liver is not necessarily a reliable indicator of the total infiltration of fat in this organ (14). For these reasons preventative action, *i.e.*, the capacity to maintain a normal fat content in the livers of several insulin-treated depancreatized dogs, was used as sole criterion for antifatty-liver activity of pancreas fractions (41). The test period was never less than four months and usually as long as five months. The entire liver was excised, ground, and thoroughly mixed, and a sample of it analyzed for total fatty acids.

The prolongation of the test period for sixteen to twenty weeks is justified on the following experimental evidence. Although fatty livers have been observed as early as three and one-half weeks after pancreatectomy, their appearance at this short interval is not a constant finding (Table IV). Livers containing in excess of 15% fatty acids were found consistently only in those animals that had been maintained for twenty weeks or longer after pancreatectomy. A positive result with this assay procedure means that the amount of a fraction fed contains enough of the antifatty-liver factor to maintain a normal fat content in the liver for long periods. Amounts of the antifatty-liver factor that are not sufficient for complete prevention of fatty livers will, of course, not be detected by this procedure.

Since assay in the insulin-treated depancreatized dog is laborious and extremely time-consuming, it is not surprising that some inves-

tigators were led to study the effects of pancreas fractions on fatty livers of normal rats induced by feeding them low-protein, low-choline, high-fat diets. Table XIII shows why this type of rat fatty

TABLE XIII
EFFECTS OF VARIOUS SUPPLEMENTS ON LIVERS OF INSULIN-TREATED
DEPANCREATIZED DOGS AND ON LIVERS OF NORMAL RATS FED
A LOW-PROTEIN, HIGH-FAT DIET

Supplement to diet	Fatty liver of insulin-treated depancreatized dog fed high-protein, low-fat diet	Fatty liver of normal rat induced by feeding low-protein, high-fat diet
Protein (meat or casein)	No effect	Prevents and cures
Free choline	Prevents and cures	Prevents and cures
Pancreas fraction 62C, 6 mg.	Prevents and cures	No effect

liver cannot be used as test object for the pancreatic, noncholine, heat-labile, antifatty-liver factor under consideration here. Fatty livers develop in the insulin-treated depancreatized dog fed an overabundance of protein, whereas the fatty liver of the rat can be prevented by the ingestion of small amounts of protein. The most pertinent evidence on this point is provided by the difference in response of the two types of fatty livers to an active pancreas fraction isolated in the Berkeley laboratories, namely 62C. The daily ingestion of 6 mg. of this fraction was sufficient to prevent a fatty liver in an insulin-treated depancreatized dog weighing approximately 10 kg. but had no effect on the fatty liver of a 200-g. normal rat. The fatty liver induced in the rat by deficient diets responds, of course, to the ingestion of such lipotropic factors as choline and methionine (36). It is therefore of interest to note here that, according to the observations of Best and Ridout (8), Aylward and Holt (5), Mackay and Barnes (37), and Entenman *et al.* (21), the antifatty-liver properties of lipocaic in the *normal rat* can be fully accounted for by its protein and choline contents.

VIII. Mechanism of Action of the Antifatty-Liver Factor of the Pancreas

Two facts stand out from the picture so far presented on the conditions concerned with the development of fatty livers in insulin-treated depancreatized dogs and in dogs deprived of the external secretion of their pancreas: First, fatty livers appear in such dogs

despite the fact that they are fed a diet not deficient in choline or in methyl precursors, but can be prevented by the addition of *extra free* choline to the diet or by the ingestion of fractions derived from the pancreas. Second, it is not the choline content of the pancreas fractions fed that accounts for their antifatty-liver action. The ingestion of as little as 6 mg. per day of fraction 62C (Table XII), which is free of choline, is sufficient to prevent fatty livers in insulin-treated depancreatized dogs weighing 10–12 kg., whereas the minimum effective dose of choline was approximately 300 mg.

Although it was pointed out quite early that the phospholipide content of blood is depressed in the insulin-treated depancreatized dog and in the duct-ligated dog maintained under conditions that promote the development of fatty livers, the significance of this observation was not fully understood until it became clear that practically no choline exists free in plasma and that nearly all choline is bound as phospholipide (49). This means that the phospholipide content of plasma is a measure of its choline content. This new observation on the nature of plasma phospholipides raised the question whether the dogs in which the external secretion of pancreas has been interfered with are suffering from a deficiency in circulating choline. That this is indeed the case is brought out in the data presented in Table XIV.

TABLE XIV
PLASMA CHOLINE OF INSULIN-TREATED DEPANCREATIZED DOG
AND OF DUCT-LIGATED DOG

Dog	Time of observation	Plasma choline, mg./100 ml.
D552	Before pancreatectomy	62.0
	28 weeks after pancreatectomy	45.0
	52 weeks after pancreatectomy	40.0
E91	Before ligation of pancreatic ducts	55.7
	22 weeks after ligation of pancreatic ducts	29.3

In this table are recorded the concentrations of plasma choline of dogs just before and at various intervals after either pancreatectomy or ligation of pancreatic ducts. Depletion of choline stores in plasma, therefore, appears to provide a reasonable explanation for the development of fatty livers in the insulin-treated depancreatized dog and in the pancreatic-duct-ligated dog. This view is in keeping with

the fact that it requires the addition to the diet of 35 mg. choline per kg. body weight per day to prevent the development of fatty livers in these dogs.

This explanation gains further support from the finding that the decrease in plasma choline does not occur when the development of fatty livers is inhibited. Thus in dogs that received daily 1 g. of AR, a dried defatted preparation of raw pancreas, in all diets given after pancreatectomy, the level of plasma choline was as high as or higher than preoperative levels (11).

Striking evidence that a principle highly active in choline metabolism is contained in the pancreas tissue has been presented. An insulin-treated depancreatized dog was maintained on a lean-meat diet for a period sufficient to permit the level of plasma choline to fall below the normal. Then 60 mg. of fraction 27C was added daily for 21 days to the diets administered. This resulted in a pronounced increase in the level of plasma choline—from 39.5 to 63.2 mg. per 100 ml. The weight of the animal increased from 7.5 to 7.7 kg. at the same time. Upon cessation of 27C treatment the plasmacholine decreased to 36.8 mg. per 100 ml., the dog's weight to 7.0 kg. A similar effect on plasma choline has been produced by feeding pancreatic juice (43).

We are now confronted with the fact that the development of the syndrome involving fatty liver and the depletion of circulating choline in these dogs is not dependent upon a lack of methyl donors in the diet. This syndrome appeared in dogs that were fed daily 500 g. lean meat, which contains about 0.5 g. choline and 3 g. methionine, an amount of lipotropic substances more than sufficient for prevention of fatty livers in normal dogs. These considerations suggest that in the dog deprived of the external secretion of the pancreas there is an interference either in the mechanism by which bound choline and methionine of the diet are made available for lipotropic purposes or in the synthesis of choline from methyl donors. Either interference could account for the decreased amounts of choline found in plasma.

But free methionine, like free choline, prevents fatty livers in the insulin-treated depancreatized dog. This is shown in Table XV. According to du Vigneaud *et al.*, methionine prevents fatty livers by supplying the methyl groups for the synthesis of choline (20). These workers have furnished indisputable proof of the transfer of methyl groups from methionine to choline in the rat, the rabbit, and man.

The finding therefore of normal amounts of fat in the livers of methionine-fed, insulin-treated depancreatized dogs can leave no reasonable doubt that the synthesis of choline from free methionine is not interfered with in the dog deprived of its pancreas (12).

TABLE XV
LIVER LIPIDES OF DEPANCREATIZED DOGS FED DL-METHIONINE FOR TWENTY WEEKS

Dog	Body weight, kg. Methionine fed				Liver	
	At start	Final	Period fed, weeks	Amount per day g.	Weight, g.	Total fatty acids, %
D515 {	7.5	5.5	20	2.1	238	3.0
D516 { Methionine fed	9.7	9.1	20	2.6	412	6.7
D517 {	7.0	7.2	20	3.0	358	2.8
D532 { Control		7.6			312	22.0
D526 {		6.0			376	17.7

In view of this action of *free* methionine, the question arose whether the fatty livers observed in depancreatized dogs fed a lean-meat diet could be overcome by increasing their protein intake. Four dogs were therefore fed daily for twenty weeks 500 g. lean meat as well as 80 g. casein. Despite the fact that this amount of protein contained 7 g. methionine, fatty livers were found in three of these four dogs. This confirms an earlier observation that the daily ingestion of 1000 g. lean

TABLE XVI
HYDROLYZED AND UNHYDROLYZED CASEIN EFFECT ON PRODUCTION OF FATTY LIVERS IN COMPLETELY DEPANCREATIZED DOGS MAINTAINED WITH INSULIN

Dog	Daily diet	Body weight, kg.		Liver	
		At start	Final	Weight, g.	Total fatty acids, %
D339	80 g.* casein	10.3	9.7	452	16.4
D380		10.9	7.6	440	17.5
D398		14.3	8.2	397	16.6
D405		17.5	14.2	550	5.9
D407		12.5	7.5	680	19.3
D586	80 g.* hydrolyzed casein	10.6	9.5	500	1.9
D590		10.5	8.7	600	1.6
D591		11.0	8.8	465	2.0

* In addition to sucrose, vitamins, and salts.

meat did not prevent the infiltration of large amounts of fat in the liver of a depancreatized dog (35).

In Table XVI the effects of feeding casein and hydrolyzed casein upon the development of fatty livers are compared. There can be little doubt from these results that the failure of 80 g. casein to prevent fatty livers is due to the inability of the depancreatized dog to release an effective amount of methionine from this casein.

The pronounced difference in the antifatty-liver action of hydrolyzed and unhydrolyzed casein justifies the conclusion that in the gastrointestinal tract of the completely depancreatized dog there is a disturbance in the mechanism whereby the methionine of protein is made available for lipotropic purposes. It was therefore proposed, as a working hypothesis, that the antifatty-liver factor of pancreas is a proteolytic enzyme (12). Such a hypothesis offers a reasonable explanation for the antifatty-liver activities of raw pancreas, pancreatic juice, and certain pancreas fractions. It also serves to explain the positive action of methionine and hydrolyzed protein as against the negative action of unhydrolyzed proteins.

But recent findings suggest that this hypothesis, at least in the simple form in which it is presented above, may not be adequate. As already pointed out in Section VII, highly active fractions which, as judged by *in vitro* tests, possess no proteolytic activity have been obtained from raw pancreas. Whether this means that pancreas contains another antifatty-liver factor, in addition to the one already postulated, remains to be determined.

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ALKALOID BIOGENESIS

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I. Introduction

The subject matter of the present essay has not been treated in a comprehensive way elsewhere in the literature. Therefore, an attempt is made herein to present a rational analysis of the problems involved and to review existing data in the light of this analysis.

The central problems of alkaloid biogenesis are, of course, the characterization of synthetic mechanisms and the discovery of relationships, if any, between these mechanisms and other physical and chemical systems within the organism. Closely linked to these problems are certain accessory and distinctly biological questions concerning loci of synthesis, patterns of accumulation, and the quantitative

effects of intrinsic and environmental factors upon production rates. For the experimenter with a chemical outlook, it is an unpleasant fact that the great majority of alkaloids are produced by plants which, because of their advanced evolutionary development, also possess the most complex physiological and structural organization. It is not surprising, therefore, that the successful study of alkaloid biogenesis should finally appear to be an interdisciplinary affair, of necessity involving a high degree of integration at the experimental level of chemistry with plant physiology and morphology. Traditionally, investigations of alkaloid biogenesis and physiology have seldom attained this unity of action. The result has been the accumulation of a relatively extensive literature written from the viewpoint either of the organic chemist or of the botanist and characterized by resort to speculation where experimental inadequacies of the divided approach made further progress impossible. Although they have contributed little to a direct understanding of alkaloid biogenesis, certain of these studies have led to the development of a sound and useful body of knowledge concerning the directions and rates of noncatalyzed organic reactions under relatively mild conditions of concentration, temperature, and acidity (see so-called "model syntheses" and "syntheses under physiological conditions" of Robinson, Julian, and Schöpf, respectively) (43,62,87,101,108-110,114-117,129,149). From the biochemical standpoint, however, such information can be regarded as indicating little more than kinetic and thermodynamic possibility of occurrence of otherwise highly improbable *in vivo* events. The basis for this seemingly paradoxical situation is to be found in currently changing conceptions of the nature of chemical processes in living things.

A. ALKALOIDS AND THE PROBLEM OF BIOSYNTHESIS

Formerly, it was widely held that chemical changes in plants and in animals very probably follow the simpler type reactions of contemporary organic chemistry. The almost universal acceptance without supporting evidence of the formaldehyde theory of photosynthesis is a case in point. Gradually, however, this view has been abandoned in favor of a new concept which regards chemical activity in the cell as mediated largely, if not entirely, by organic catalysts. At first, emphasis was placed upon the down-grade aspects of enzymic activity such as those found in the digestion and oxidation of carbo-

hydrates, fats, and proteins. The importance of enzymes in biosynthesis has only recently been recognized, and it is an interesting commentary that such recognition has come about principally through the application of uniquely biological concepts (*i.e.*, the principles of genetics) to biochemistry. The work of Beadle and Tatum and their school has demonstrated, for instance, that a one-to-one relationship holds between genes and specific reactions in the synthesis of certain vitamins and amino acids in the mold *Neurospora* (6,133). Such findings as those of Umbreit, Wood, and Gunsalus (136) and Schweigert (118) appear to support this conclusion. According to Beadle (6), the hypothesis upon which his interpretations are based may be expressed as follows: "In determining the specific chemical and perhaps physical configurations of protein molecules, genes directly determine enzyme specificities and thereby control in a primary way enzymatic syntheses and other chemical reactions in the organism." While it is not to be presumed that the simple one-to-one relationship between genes and reaction steps as developed by Beadle can explain either all cases of biosynthesis or all instances of genic control, it nevertheless is steadily becoming more apparent that the underlying assumption of enzymic regulation of metabolic pathways can no longer seriously be challenged.

Existing evidence which can be used to relate alkaloid biosynthesis to enzymically catalyzed sequential reaction series is painfully meager. The compelling fact remains, however, that not only the ability to synthesize a given alkaloid but also the actual rate at which it is produced are controlled by hereditary means (71,126). Thus, it seems reasonable to assume the occurrence of some type of gene-enzyme-reaction relationship similar to that found by Beadle and Tatum in *Neurospora*.

Michaelis (90) and Kalckar (63) have given emphasis to the fact that enzymes may raise to a level of kinetic importance reactions which, although thermodynamically possible, either do not occur to an appreciable extent or are superseded by other more rapid reactions in the absence of such enzymes. Thus, biological reaction pathways may be considered to be potentially unique. Indeed, the relative frequency with which these pathways (*e.g.*, glycolysis) fail to coincide with what might be predicted on the basis of the noncatalyzed type reactions of organic chemistry alone would seem to suggest a fairly widespread realization of the uniqueness principle.

The probability of enzymic mediation of alkaloid synthesis by way of highly specific reaction steps would seem to render irrelevant the many schemes based upon purely thermal processes by means of which alkaloid biogenesis has been "explained" in the past. For this reason attention will be directed in the remaining pages to the results of those investigations which have sought to deal directly with the problem at the *in vivo* level.

B. PHYSIOLOGICAL FUNCTIONS OF ALKALOIDS

There are three serious deficiencies in alkaloid biochemistry that may be ascribed directly to the historical lack of a unified approach. One of these is the absence of evidence that would relate alkaloids to definite biochemical or biophysical functions and thus explain their significance in the metabolism of the plants that produce them. A second is failure to establish direct connections between alkaloid production and catabolic processes on the one hand and/or anabolic processes on the other. In other words, up to the present it has still been impossible either to discover whether alkaloid production is the result of synthetic or degradative activities in the empirical sense or to learn anything about the relationship, if any, of alkaloids to the general physiological chemistry of the organism. Notwithstanding the absence of definitive evidence bearing upon these points, there have grown gradually over a period of time a number of speculative notions some of which are still widely accepted in lieu of anything more concrete. By and large, the impression seems to prevail that alkaloids, being nitrogenous, must be produced parallel with or as a result of some phase of protein metabolism, either protein synthesis or protein breakdown (2,16,43,57,139). It may be noted, parenthetically, that no one seems to have considered it necessary to postulate a similar relationship for such closely related compounds as the vitamins. Again, because it has been impossible thus far to discover an *in vivo* function for alkaloids and because they frequently accumulate in barks, seed coats, and other terminal structures, it has been assumed that these substances represent essentially waste products of metabolism formed as a result of irreversible reactions, usually unavailable for further exploitation by the plant cell, and hence discarded by deposition in tissues where metabolic activity is at low ebb (16,81,139).

With respect to the third of these deficiencies, from the enzymic

point of view there is a wide discrepancy between the activities of many alkaloids for plant and for animal tissues. In a large number of cases alkaloids when introduced into the animal body in minute amounts exert powerful physiological effects which are almost certainly concerned with some type of interference with or inactivation of enzyme systems (41,50,61,145,150). On the other hand, the presence or absence of the same alkaloids in the tissues of the plants that produce them has always seemed to be a matter of relative indifference. For instance, it has been possible in the author's laboratory to produce tobacco leaves experimentally that contain a wide range of nicotine contents from extreme values of 8 to 12% of dry weight down none at all without any externally visible effects upon growth rate, morphology, or ability to reproduce (unpublished observations). As will be shown later, however, there is room for belief that failure to detect any physiological activity whatsoever in plant tissues is the result more of faulty experimentation than of actual lack of such activity. For this reason and because of the close structural relationship between alkaloids, vitamins, antivitamin, coenzymes, and specific drugs, a careful examination of this general problem in the light of recent advances in enzyme chemistry seems greatly to be desired.

C. THE PROBLEM OF EXPERIMENTATION

A survey of the literature reveals certain defects in experimental design and errors in basic assumption which have contributed substantially to the sterility of many earlier investigations of alkaloid biogenesis. In brief outline these are: (1) the tendency of most investigators to express analytical data indiscriminately in terms of per cent of fresh or dry weight rather than in absolute quantities per organ or per plant; (2) failure to recognize the importance of determining the sites of synthesis of the alkaloid in the plant body as opposed to sites of accumulation; (3) the use of nonspecific methods for quantitative chemical estimation of alkaloids in plant tissues without independent and confirmatory evidence; and (4) the tendency to adopt *a priori* assumptions concerning (a) the intermediary relationships of alkaloid formation to protein metabolism (100,111,135), (b) the role of photochemical reactions in the production of the fundamental heterocyclic ring structures (4,5,51,98,127), (c) the nonnecessity for enzyme participation in the sequence of chemical steps leading to synthesis (100,101,108,110,114,129), and/or (d) the probability

that the green leaf is the seat of synthesis of most of the active plant principles (15,17,24,93,132).

In the light of all that has gone before, the rational investigation of the biogenesis of any individual alkaloid might therefore be expected to proceed somewhat as follows:

1. Determination of the locus of synthesis in the plant body to the end that suitable tissues or organs might be selected for use in subsequent experimentation. A great many investigations of alkaloid synthesis have been reported in which the tissues employed were actually incapable of forming the alkaloid in question (17-21,23,24,40,54,57,59,60,77,88,89,93).

2. The identification of chemical intermediates and of the catalysts concerned in the sequential steps leading to synthesis.

3. Characterization of other physical and chemical systems associated with the process of alkaloid formation or with the activity of the newly synthesized product and the way in which these systems are integrated with the general physiology and biochemistry of the plant.

4. Evaluation of the effects of internal (*i.e.*, genetic, hormonal, etc.) and of external (environmental) variables upon the differential rates of growth and alkaloid synthesis.

In recent years gratifying progress has been made as a result of the systematic investigation of alkaloid biogenesis from the points of view outlined above. The way now seems clear, therefore, for a direct and successful attack upon the principal problems of biosynthesis and physiological function.

Before undertaking a discussion of these advances, however, it seems advisable to call attention to the fact that alkaloids probably do not represent a homogeneous group of substances from the physiological or even from the biochemical standpoint. Up to the present time the classification has been primarily of use to the organic chemist and has served as a repository for those compounds which can be considered as derivatives of pyridine, quinoline, isoquinoline, etc., for which no biological significance is known (*e.g.*, pyridoxine is a derivative of pyridine but is not classed as an alkaloid because of its known role in enzyme systems and as a vitamin). It might reasonably be expected, therefore, that ultimately the various alkaloids may be reclassified on the basis of their metabolic activity into such categories as, for instance, vitamins, coenzymes, antivitamins, antienzymes, and

even outright waste products. In this event, the present arbitrary distinctions between such compounds as nicotine, nicotinic acid, and penicillin may eventually disappear.

To the same extent that uncertainties of definition introduce uncertainties of biochemical interpretation, so also do they necessitate caution in attempts to derive generalizations from existing data that will hold true for diverse groups of alkaloids and for different genera and species of plants. Therefore, in the discussion that follows, it is well to bear in mind that the points of similarity between independent systems may be entirely fortuitous.

II. Loci of Alkaloid Synthesis

The plant body is rather simply divided into three major external organs—the root, stem, and leaf. For the purpose of exploratory experimentation it is usually necessary that localization of an alkaloid synthetic mechanism be attained only with respect to organs rather than to the tissues that compose the organs. Eventually, even the latter information is also necessary, however. The demonstration of localized synthesis in root, stem, or leaf would be relatively simple but for the complication introduced by possibilities for alkaloid migration from the site of initial synthesis to other portions of the plant body where accumulation may ensue. It therefore becomes necessary to determine whether in a given case the presence of an alkaloid in a plant organ is the result of formation *in situ* or of translocation followed by secondary accumulation. The choice between these two possibilities may be still further complicated by degradations or other secondary chemical modifications of the alkaloid molecule subsequent to its deposition in the area of final accumulation. The question thus arises with respect to the kinds of experimental evidence that may be of value in the clear-cut separation of these variables.

A. SOURCES OF EXPERIMENTAL EVIDENCE

Three investigational procedures have contributed most effectively to the identification of synthetic loci. These are: (1) the detailed description of accumulation patterns in the plant body at all stages of growth and development from the ripe seed to the senescent individual; (2) the culture of isolated organs *in vitro*; (3) the preparation of transplantations (*i.e.*, grafts in horticultural terminology) between species of plants that produce alkaloids and those that do not. A

brief characterization of each of these procedures together with an estimate of its experimental value and limitations is presented below as an aid to the interpretation of discussions that are to follow.

Distribution Patterns in the Plant Body. The determination of alkaloid accumulation patterns in the plant body is capable of yielding much useful information about (a) areas of preferential accumulation, (b) correlations between intensity of synthesis and rate of growth, (c) positions of prior appearance in point of time, and (d) general drifts in over-all production rates with time and stage of development. From the standpoint of localizing synthetic mechanisms, however, such information is incapable of differentiating between accumulations that are a result of synthesis *in situ* and those that are a result of translocation and secondary deposition. If, for instance, it became established that a given alkaloid appears in the root tip of the seedling prior to its appearance in the stem (plumule), it cannot be safely argued that the root tip is indicated as the probable seat of synthesis. As a consequence of the occurrence of translocation, it can be admitted only that, within the limits of sensitivity of the method employed for detection, the alkaloid in question can be observed to accumulate first in the root tip. The locus of synthesis, if there is one, may with equal force of logic be assumed to exist in some other portion of the seedling including, *e.g.*, the stem tip. Indeed, since the apical meristems of root and stem are centers of the most intense immigration and assimilation of protoplasmic constituents, a reasonable argument might be advanced for the extraneous origin of the alkaloid concentration that appeared in this hypothetical root tip. A second example is also useful in evaluating some of the earlier contributions to this field. In this case, it is assumed that synthesis *in situ* is responsible for the development of comparatively high alkaloid concentrations locally. By relatively slow processes similar to diffusion it is then imagined that the alkaloid migrates to other portions of the plant body where its concentration is always lower than that maintained at the point of origin by continued production. Obviously, an exactly opposite explanation may also be given by assuming that the alkaloid is removed from the seat of synthesis almost as fast as it is formed and translocated via the xylem or phloem to some tissue or organ which serves as a "sink." In any event, the usefulness of most of the available data on alkaloid distribution patterns in the plant body is greatly limited by the absence of figures for

the alkaloid content of roots and root systems. One excellent reason for this deficiency is the virtual impossibility of recovering the roots of a plant *in toto* from soil or from the medium upon which the plant was grown. The other reason stems from the preoccupation traditionally exhibited by physiologists and biochemists with the green plant leaf (see above).

The above discussion is based upon the assumption that the chemical methods utilized in the determination of alkaloid accumulation patterns are dependable. In actual practice, however, few of the methods used for the quantitative estimation of alkaloids in plant tissues possess a high degree of specificity. For instance, "nicotine" has been determined by precipitation with silicotungstic acid for many years; yet only recently has it become apparent that, in certain rather important cases involving cigarette tobaccos, the principal alkaloid involved has been nor nicotine rather than nicotine (86). Such pitfalls indicate the necessity for parallel and independent evidence of identity when one of the usual nonspecific titrimetric or gravimetric methods is used. Alkaloids have been detected qualitatively and their relative concentrations estimated visually by the application of precipitating or staining reagents to thin sections of plant tissues under the microscope. The most popular and widely used reagent is a solution of iodine in potassium iodide (14,56,81). Notwithstanding the fact that this solution stains starch and proteins in addition to precipitating a variety of bases, distribution patterns reportedly obtained with its use seem to agree in general with those determined by macrochemical means.

Finally, attention must be drawn to a still more fertile source of error in the study of alkaloid distribution patterns. This is the tendency, deeply rooted in biochemical practice, of expressing analytical data in terms of concentration in per cent of alkaloid in fresh or dry plant tissue. Thus, if the percentage of alkaloid is greater in the younger leaves of a plant than it is in the older ones, for instance, the ambiguous statement is made that the alkaloid "content" of the younger leaves is greater. Such conclusions pass into the literature and, removed from the original context where the exact meaning of the term "content" is no longer clear, are cited repeatedly as providing evidence for entirely erroneous deductions concerning, *e.g.*, comparative rates of alkaloid production or accumulation by young and old leaves. The fact is that the dry weight of plant tissues is deter-

mined almost solely by the rates of production of cell wall materials such as cellulose and lignin and of protoplasmic protein. Fresh weight involves in addition a still more variable and erratic quantity representing the mass of water that happens to be present in the tissues at the time of sampling. Now, there is overwhelming evidence available in the literature to show that absolute rates of alkaloid production bear little if any direct mathematical relationship to rates of cellulose and lignin formation, to protein synthesis, or to water absorption (64,93,140); nor is there any *a priori* reason for thinking that they should. Therefore, the persistent use of "percentage of dry weight" as a basis for expression of analytical data in studies of alkaloid distribution can receive little or no justification outside of the possible indication of suitability of certain plant materials in the bulk for commercial processing. At the center of the problem of alkaloid biogenesis at the present time is the need for careful and accurate measurements of the absolute rates of formation and accumulation of alkaloids by the biological unit (*i.e.*, the root, stem, leaf, or plant as a whole) under a variety of experimental circumstances. This approach involves not only a consideration of the concentration of the alkaloid in a particular organ or tissue but also the total mass of that organ or tissue. Under these conditions, the conclusions referred to above in connection with alkaloid accumulation in leaves are very frequently reversed, and the alkaloid content of older leaves on an absolute basis may greatly exceed that of younger leaves.

The use of absolute quantities in the investigation of production and accumulation patterns is not without pitfalls for the unwary, however. Where the experiment requires taking successive or serial samples of initially identical total alkaloid content, great care must be exercised to insure that these conditions are actually met. In most cases, unless the size of the sample is so great as to become unwieldy identical contents are almost impossible to obtain by random selection. Likewise, difficulties are to be encountered in comparing organs or tissues of different internal structure, age, size, or previous history; and these differences must be emphasized and, if possible, evaluated quantitatively in the interpretation of the experimental data. Although it has not yet reached that phase of development alkaloid biochemistry must eventually come to grips with the problem of the ultimate basis for comparisons among tissues that differ in such fundamental properties as cell size, number of chromosomes, etc.

as occur, for instance, in hypertrophied *vs.* normal and tetraploid *vs.* diploid cell types.

Culture of Excised Organs. One of the most useful tools in the study of alkaloid production and of biosynthesis in general is the culture *in vitro* of excised portions of the plant body free from the complications introduced by attachment to other organs via the vascular system. Leaves, stems, and leafy shoots have long been cultured for limited periods of time by immersing the cut ends in water or in aqueous solutions of various hypothetical intermediates in alkaloid synthesis (23,24,39,54,67,142). Under these conditions the tissues remain turgid for a few days, and presumably normal processes are operative for at least a short time. Quickly, however, protein digestion and amino acid oxidation assume a dominant role (142), and the metabolism of the excised organ becomes essentially pathological. Within the short initial period of "normal" metabolism, it is sometimes possible to determine whether or not these organs are capable of synthesizing and of accumulating alkaloids under the experimental conditions imposed. As is indicated above, however, it is frequently very difficult or even almost impossible to detect factually significant changes in the alkaloid content of organs which, as a result of their attachment to the parent plant prior to excision, unavoidably contain appreciable and widely variable amounts of alkaloids at the beginning of the culture experiment.

A closer approach to the real culture of the aerial portions of green plants under sterile conditions in glass has been obtained by Loo (80). Although this method has not yet been applied to the problem of localizing alkaloid synthetic mechanisms, there is every reason to believe that it should yield evidence of great value especially in those cases where small residual capacities for alkaloid synthesis on the part of leaves and stems are claimed by some investigators.

Excised roots have provided the most useful organ culture in studies of biosynthesis to the present time. These organs will in many cases grow rapidly and submit to repeated and indefinite subculturing when supplied only with sugar, inorganic salts, and a few vitamins (107,146,147). They are obtained readily in a sterile condition by treating the seeds with sodium hypochlorite in dilute aqueous solution or with alcoholic mercuric chloride prior to germination. Based upon the experience with these cultures to date it has been possible to demonstrate that the roots of some plant species not only produce

and accumulate alkaloids in their tissues but also excrete them in still larger amounts to the external medium. It is assumed that this type of excretion represents merely an escape of the alkaloid from the cut basal end of the root in the absence of organic connection with the vascular system of a leafy shoot. Presumed intermediates in alkaloid synthesis can be added at any time under aseptic conditions, and production rates can be very easily determined either by drawing off aliquots of the culture fluid or by sacrificing replicated culture sets. Finally, in contrast with the usual water cultures of excised leaves referred to above, the demonstration of alkaloid synthesis and accumulation is not made equivocal by major sampling difficulties or by deep-seated disturbances of tissue metabolism. Indeed, the only other type of culture that can be compared with excised roots in their potential utility for studies of alkaloid formation is that of excised stem tips as described by Loo (80).

The principal limitations to the use of excised root tip cultures are the facts that (1) "normal" growth has not been achieved in the sense that, under the conditions of culture, growth in diameter due to lateral cambium activity does not occur and (2) no one has yet successfully grown through an indefinite series of subcultures the roots of a monocotyledonous plant. Such limitations do not seem to be serious in the present connection.

Transplantations (Grafts). The problem of identification of synthetic loci has been greatly simplified in the case of certain alkaloids by the interchange of root and shoot systems between one species of plant which produces a given alkaloid and another species which does not. The great advantage of this procedure is that it allows for visibly normal development of the organ under study, free from the complication of alkaloid immigration from other parts of the plant body. The appearance anywhere in the grafted plant of an alkaloid characteristic of the species under study constitutes excellent evidence for the synthetic capabilities of that species, since it may be shown that the presence of the alkaloid in the normally alkaloid-free member of the graft combination is a simple consequence of translocation. The practicality of grafts as experimental material is made possible by the ready formation of wound tissue and the establishment of vascular connections between stock and scion (*i.e.*, root and shoot components, respectively) in those cases where the phylogenetic relationships between the two species or genera involved

are not too distant. Indeed, the stimulus for the earliest investigations of alkaloid distribution between stock and scion in grafted plants was the question, seriously debated in those days, whether organic substances could freely pass across a graft union involving different species or genera (*i.e.*, so-called heteroplastic grafts).

In practice, the development of a normally alkaloid-containing shoot on a nonalkaloid-producing root may result in either of two eventualities. In one, the scion fails to accumulate alkaloids and therefore becomes essentially alkaloid free as it grows. It may be assumed that the leaf and stem of the scion species do not carry out total alkaloid syntheses in the intact plant. If, on the other hand, the scion does contain alkaloids, then it may likewise be assumed that the stems and/or the leaves of the species under examination normally produce the same alkaloids. In like manner, if normally nonalkaloid-containing scions are grafted to the roots of an alkaloid-producing species, two possibilities again emerge. If the scions do not contain alkaloids and if the roots are also free of them, it seems probable that the stock species does not normally produce alkaloids by total synthesis in the roots. Conversely, if the stocks and/or the scions do contain alkaloids it can be assumed that these alkaloids arise in the root species and have appeared in the scions as a result of translocation.

Although the interpretation of results obtained with grafted plants is usually straightforward, there are cases known in which the accumulation of alkaloid in the normally alkaloid-free component is associated with some secondary modification such as the removal of a methyl group or the probable hydrolysis of an ester linkage (see below). Otherwise, there are no fully convincing instances known to the author which require for their interpretation the acquirement of new synthetic powers (71,83,119–123) or the loss of old ones (7,54,71,83,119–123) under the influence of the changed environmental conditions produced by grafting. The first of these views is LaMarckian in the extreme and, in spite of adroit defense by its proponents, has not yet received even reasonably well-documented support. An excellent example of one such claim and the faulty experimental structure upon which it was based is presented in a later section (anabesine, see page 229).

A loss of synthetic power under the influence of an extraspecific graft component would seem to lie more within the realm of possibility, since it is, at first thought, conceivable that a necessary factor

for enzyme activity may not be provided by this component, or that an enzyme inactivation might occur under the influence of some substance or substances obtained from the graft partner. Such explanations for the failure of graft leaves, for instance, to accumulate alkaloids have not received direct experimental confirmation, and their probability of correctness is greatly reduced by a number of considerations. First, since the protoplasm of the cells of the higher plant is made from simple raw materials brought up from the root plus sugar from the leaf, it is difficult to imagine how such an integral part of the protoplasm of a cell as an enzyme necessary for alkaloid synthesis could be lost permanently by virtue of leaf growth and development in connection with the root of a foreign species. If one enzyme or protein is altered or lost altogether under such circumstances, one would expect that more such instances would occur and that real mutations could be obtained by grafting alone. There is no evidence that the latter possibility is true, and there is, indeed, ample evidence in the case of the tobacco plant (unpublished data) that the failure of graft leaves to accumulate nicotine is due to something other than the permanent loss or inactivation of an enzyme system due to grafting (*i.e.*, seeds that are produced in association with such alkaloid-free leaves germinate to yield offspring that contain normal amounts of nicotine). In the same connection it may be noted that normally nonalkaloid-containing leafy shoots, which contain large amounts of nicotine as a result of having been grafted to tobacco roots, also produce seeds that reproduce the original scion type absolutely (*i.e.*, tomato seedlings free from tobacco alkaloids). Secondly, it has been possible to obtain alkaloid-free leaves and stems by grafting scions of the same species of alkaloid-containing plant upon a variety of rootstocks representing several different species and even genera. It seems improbable that these different rootstocks would be capable of exerting some common and specific inhibitory action upon one and only one enzyme system in the leaves.

Definitive evidence for the possibility of loss or inactivation of a synthetic mechanism under the influence of grafting can at the present time be secured only by the use of cultures of excised root tips, stem tips, or leaf primordia. In those instances in which such comparisons have been made (27-29) the results of culture experiments have coincided exactly with the results of grafting.

There remains the possibility that roots or shoots which fail to ac-

cumulate alkaloids in culture or in the grafted condition may still be able to carry out to a very limited extent the final steps in alkaloid synthesis if provided with suitable intermediates by their own complementary organs or by the experimenter. This problem is of some importance from the point of view of completing the picture of alkaloid production by the intact plant, but its immediate resolution is of no great urgency in connection with the principal problems of alkaloid biosynthesis. On the other hand, it has been pointed out that the accumulation of an alkaloid in the normally alkaloid-free component of a graft combination may be a result of the ability of this component to assemble the final alkaloid molecule from its immediate precursors. While there are theoretical objections to a view which would permit such activity on the part of plant tissues that do not contain the enzyme complement necessary for total synthesis of the alkaloid, experimentally it is possible to test the view by examining the exudate that bleeds from the xylem and phloem of properly excised stems of alkaloid-producing species. In all cases to date, the alkaloid has been isolated or at least qualitatively identified in such exudates in the complete state of assembly.

B. ALKALOIDS PRODUCED IN ROOTS

Nicotine. Early investigations of the distribution of nicotine among the different organs of the tobacco plant (*Nicotiana tabacum* L.) at various stages of growth and under a wide variety of experimental conditions clearly demonstrated the need for an inquiry to determine whether or not nicotine synthesis is localized within the plant body. Some of the facts pointing in this direction are: (1) the lack of an obvious relationship between the growth rate of stem and leaves and the rate of nicotine accumulation in these organs (84,93,140); (2) the absence of a consistent relationship between the nitrogen metabolism of the tobacco leaf and its nicotine content (25,93,140,142); (3) the smooth and uninterrupted accumulation of nicotine in the leaves during the growth of the tobacco plant and the sudden stabilization of nicotine content when the leaf is removed from the plant and either cultured for several days in various solutions or placed in the curing shed (32,93,142); (4) the renewal of nicotine storage in excised tobacco leaves when these leaves have been induced to form roots at the petiolar ends (26); (5) the preferential

accumulation of nicotine in the structurally leaflike cortex of the green tobacco stem (stalk) with its loosely arranged cells and stomatal apparatus and in those areas of the individual tobacco leaf (the tips and margins) in which transpiration might be expected to occur with greatest intensity (1,26); and (6) the failure of those experimental variables that most directly affect the aerial parts of the tobacco plant to alter appreciably the rate of nicotine accumulation in the leaves (37,93). In addition, the author has detected an inverse correlation between the dry weight and nicotine concentration of the stems and leaves of the same variety of tobacco when grown in consecutive crops throughout the year in a greenhouse under the influence of seasonal changes in light, temperature, etc. (unpublished data).

The first attempts to demonstrate localized synthesis of nicotine in the tobacco plant were carried out by Meyer and Schmidt (88,89). Tobacco scions were grafted to rootstocks such as a low-nicotine-containing species of *Nicotiana* and to potato. The data clearly show that nicotine all but disappeared from the tobacco scions, but the quantitative value of the analytical figures is greatly reduced by the fact that the latter were obtained by a relatively insensitive method of analysis (titration) which did not differentiate between nicotine and small amounts of ammonia and other volatile bases. The same may be said of the results of a study of some reciprocal grafts between the low-nicotine *Nicotiana affinis* and *N. tabacum* by Grafe and Linsbauer (40), for here again, following an ether-alcohol-potassium hydroxide extraction of the dried tissues, nicotine and accompanying bases were distilled with steam and titrated with 0.1 *N* acid to an end point with rosolic acid indicator. In each of the works cited the investigators assumed tacitly that nicotine is formed in the leaves of the tobacco plant and is then moved downward gradually into stem and root. Hence, their primary concern was with the apparent alkaloid content of the lowermost organs of their grafted plants, and the significance of the failure of nicotine to accumulate in normal amounts in the leaves of the tobacco scions eluded them altogether. Javillier (59,60), employing the gravimetric silicotungstic acid method and steam distillation, was unable to detect nicotine in the tubers of potato plants to which tobacco scions had been grafted and thus indicated that the downward migration of nicotine from tobacco leaves to potato roots claimed by Meyer and Schmidt on the basis of unsatisfactory methods of analysis did not actually occur. It is most unfor-

tunate that Javillier did not give greater attention to the tobacco leaves of this graft combination.

In 1936 Nath reported the results of experiments in India (95) in which it was found that tomato scions grafted to tobacco stocks ultimately contained 1.27% nicotine, while the reciprocal type of graft combination yielded only about 0.35% nicotine in the tobacco scion and none in the tomato stocks. The same variety of tobacco in the nongrafted state contained 3.36% nicotine. Hasegawa (42) performed similar experiments in Japan and arrived at essentially the same results. The latter author pointed out that confirmation of his results would result in a reversal of the conventional point of view regarding nicotine in the physiology of the tobacco plant inasmuch as the alkaloid must now be regarded as an actively translocated form of nitrogen. Hasegawa was apparently the only one of the earlier workers to suspect the real meaning of the results of these grafting experiments. It is quite clear that he regarded nicotine translocation in the tobacco plant to be unidirectional and acropetal in nature. Bernardini (7) reported confirmatory results from Italy and emphasized that he had obtained an essentially nicotine-free tobacco by grafting commercial varieties to tomato rootstocks.

The Russian school under the influence of Kostoff and Shmuck (70,71,119-123) undertook an extensive study of alkaloid occurrence and distribution in grafts involving not only commercial varieties of smoking tobacco but also the insecticidally important *Nicotiana glauca* which is characterized by the predominance of an alkaloid anabasine, an isomer of nicotine. When scions of *N. tabacum* and *N. rustica* were grafted to rootstocks of nightshade (*Solanum nigrum*), Jimson weed (*Datura stramonium*), and to tomato (*Lycopersicon esculentum*), nicotine could not be detected in any of the scion parts. When the reverse grafts were prepared, nicotine was found in all scions regardless of the species represented (123). These results were interpreted to mean that *Solanum*, *Datura*, and tomato leaves, respectively, acquired the ability to synthesize nicotine under the influence of the tobacco root. It need be remarked here only that such an interpretation of the results of grafting experiments cannot now be accepted for the reasons outlined earlier. The results obtained with reciprocal grafts of *N. glauca* and *N. tabacum* are complicated and are discussed in a succeeding section.

A series of experiments of similar nature was performed in the

author's laboratory in the University of Missouri. Tomato was used as the nonalkaloid component of the graft combinations. Actually, tomato does contain an alkaloid, solanine, which is the same as that found in the potato. The chemical and physical properties of solanine are such, however, that little or no interference is to be expected with either the synthesis or the analytical determination of nicotine provided the latter is removed from the tissues by a preliminary steam distillation. Tomato scions grafted to Turkish tobacco rootstocks accumulated nicotine as they grew in much the same manner as normal tobacco plants (26,27). That is, not only was total accumulation by the scion a smoothly continuing process but also the distribution of the alkaloid between the various parts of the aerial shoot resembled that in the intact tobacco plant (Table I). Even the tomato fruits contained small amounts of nicotine isolated and identified as the crystalline dipicrate. It has been remarked earlier that tomato plants grown from the seed of nicotine-containing tomato fruits were themselves nicotine free (unpublished data). The only visible evidence in grafted plants of an influence of stock on scion was the progressive killing of the lowermost and oldest tomato leaves as their nicotine content rose to relatively high values.

TABLE I
ACCUMULATION OF NICOTINE IN TOMATO SCIONS GRAFTED ON TOBACCO STOCKS
AND IN TOBACCO SCIONS GRAFTED ON TOMATO STOCKS (27)

Type of scion	Nicotine in milligrams per scion (mean of four scions)			
	Number of days after preparing grafts			
	0	31	58	84
Tomato	0	55	258	571
Tomato	0	38	135	501
Tobacco	1.3	2.6	4.1	2.5

In the same experiments, tobacco scions grafted to tomato rootstocks contained nicotine only in the lowermost leaves (which had been present in juvenile form in the original tobacco scion) and then only in amounts of an order of magnitude comparable with those of the scions used at the time of grafting. It is obvious that the nicotine present in these young leaves at the time of grafting merely remained there unaltered and could be recovered from the same leaves at the end of the growth period. The leaves in the center of the

tobacco stem or stalk contained no nicotine whatsoever by the silicotungstic acid turbidity test. The uppermost leaves and stems together with the inflorescence contained a base or mixture of bases which gave oily picrates that refused to crystallize and therefore probably did not contain nicotine. Incidentally, since these bases could be precipitated with silicotungstic acid and since they were high boiling, they would automatically be determined as nicotine in any of the current procedures for the quantitative estimation of this alkaloid. It is therefore again emphasized that questions involving the presence or absence of nicotine itself in graft combinations must be answered by the direct isolation of the alkaloid in addition to the use of indirect methods of analysis.

It is interesting to note that tobacco leaves from plants grown on tomato roots very closely resemble the leaves of intact plants in external appearance. Yet they are highly susceptible to attack by aphids and other sucking and leaf-chewing insects. Again, although there is little reason to suspect that they might do so, plants grown from the seed produced by nicotine-free tobacco scions contain nicotine in normal amounts (unpublished data). It may be noted also that the above results obtained with tomato as the graft partner have been substantiated wholly or in part by the use of *Datura*, eggplant, and petunia in reciprocal grafts with tobacco. Since the above results point unanimously to the conclusion that nicotine originates solely in the root of the tobacco plant and is translocated from there upward to accumulate in stem and leaves, a number of ancillary experiments have been performed to test this hypothesis.

First, if nicotine were actually synthesized only in the root and then translocated via the xylem to the shoot, the tips of tobacco stems inserted laterally into tomato stems as nicotine donors should induce an asymmetrical accumulation of alkaloid in the leaves of the tomato "acceptor" that lie above the graft union. This assumption proved to be correct, for in one case 84% of the total amount of nicotine to appear in the tomato acceptors was found in the 29 leaves which were located in the sector of the stem immediately above the point of insertion of the tobacco donor. The remaining 16% of the alkaloid was distributed among the other 70 leaves from all other sectors of the tomato stems (27). Secondly, if nicotine is actually preformed in the root and translocated as such to stem and leaf, it should be possible to detect the alkaloid in the transpiration stream during passage

of the latter through the vascular tissues of the tobacco stem. Such is indeed the case, for nicotine was isolated as the dipicrate from the liquid which bleeds from the cut surfaces of the vascular tissues of the severed tobacco stems (26).

Final proof for the localization of the nicotine synthetic mechanism was eventually obtained by the isolation of nicotine dipicrate from the tissues and spent culture fluids of excised tobacco roots in sterile culture on a medium of inorganic salts, sucrose, and yeast extract (27). Some of the data are recorded in Table II. For the first time, therefore, the investigator of alkaloid biosynthesis has been provided with an experimental object possessing known synthetic capabilities and readily submitting to indefinite culture under sterile conditions.

TABLE II
NICOTINE PRODUCTION BY EXCISED TOBACCO ROOTS IN STERILE CULTURE (27)

Period of culture, days	Approximate nicotine production, μ g.	
	Per culture	Per 100 μ g. dry roots
35	62	—
41	73	—
52	31	1
55	136	2
73	124	1.2
78	155	—
129	291	2
163	827	2.5

The question whether leaves and stems contribute to the total nicotine output of the plant as a whole is answered in the negative by the following facts: (1) grafted leaves in the experience of the author have never shown a nicotine content greater than could be accounted for on the basis of carry-over from the original scion; (2) when tobacco leaves are removed from intact plants and cultured in water or cured, their normal rate of nicotine accumulation is invariably interrupted, and an actual loss of about 10% of the amount originally present may occur (93,142); and (3) the vigorous succulent shoots that develop from lateral buds on excised and defoliated tobacco stalks that have been held in a moist chamber contain no detectable quantities of nicotine or other alkaloids (27). Chaze's extensive histochemical studies of the early appearance of nicotine in the ex-

panding vacuoles of young cells throughout the aerial shoot must be interpreted as indicating accumulation rather than *in situ* synthesis for reasons already discussed (10-14).

Finally, therefore, the process of nicotine formation and accumulation within the tobacco plant may be characterized as follows: (1) nicotine is synthesized solely in the tobacco root and not at all in leaf or stem; (2) the great bulk of the newly formed nicotine is transported via the xylem upward to stem and leaf where it accumulates during the life of the plant; and (3) leaves as accumulators, in contrast to roots as producers, quickly acquire a substantially higher and continually increasing content of nicotine. A rational explanation is thus provided for the fact that the sites of greatest alkaloid accumulation in the plant body are not necessarily identical with the sites of most intense alkaloid synthesis.

One of the striking facts relating nicotine formation in *Nicotiana tabacum* and in *N. rustica* (45,123) to the production of the same alkaloid in other species and even genera is the apparently universal restriction of the synthetic mechanism to root organs. The wild species *N. glauca* and *N. glutinosa* have been shown to produce nicotine only in their root systems (29,30). Likewise, the remarkable Australian tree, *Duboisia myoporoides*, has been shown to donate nicotine to tobacco scions grafted upon its roots (49). Great interest should attend any effort to identify the locus of nicotine formation in such other widely unrelated species as milkweed (*Asclepias*), the scouring rushes (*Equisetum*), and the club mosses (*Lycopodium*), all of which have been reported to contain nicotine (85).

The salient features of the above outline of experimental evidence have been confirmed independently by the work of Hieke (45), Mothes and Hieke (94), Pal and Nath (97), and Neagu (96).

Anabesine. This alkaloid is a structural isomer of nicotine and has been isolated from Kentucky tobacco by Späth and Keszler (128). It apparently occurs in most cultivated tobaccos in extremely minute amounts if at all. It occurs in appreciable concentrations, however, in the wild *Nicotiana glauca* and in the Russian weed *Anabasis aphylla* (43). Anabesine occurs in all parts of the plant body of *N. glauca* but in concentrations that are notably lower than is usually the case for nicotine in *N. tabacum*. The latter situation may be a consequence of the fact that the vegetative tissues of *N. glauca* are diploid in nature while those of *N. tabacum* are tetra-

ploid (29,32,71,125,126). Shmuck and associates have studied the accumulation of anabesine in reciprocal grafts of *N. glauca* with tomato. In contrast with the results earlier obtained with nicotine, anabesine appeared in *N. glauca* scions grafted to tomato roots as well as in tomato scions grafted to *N. glauca* roots (123). This finding was confirmed in the author's laboratory (28,29), and the additional information was obtained that, although anabesine was apparently synthesized in the *N. glauca* scions in normal amounts, none of the alkaloid was translocated downward to the tomato stock, which as a consequence remained alkaloid free. Shmuck interpreted his data to mean that the tomato scions acquired the property of anabesine synthesis as a result of their development upon *N. glauca* stocks. For reasons outlined earlier, it is more likely that anabesine is synthesized in both root and shoot of the intact plant and that the anabesine content of the shoot is derived in part from translocation from root and in part from synthesis *in situ*. The ability of roots to synthesize the alkaloid has been confirmed by the isolation of anabesine dipicrate from the tissues and spent culture fluids of excised roots of *N. glauca* that had been grown in sterile culture (28).

Similar results have been obtained by employing reciprocal grafts between tomato and the hybrid *N. tabacum* \times *N. glauca* (29). It thus seems clear that the capacity for anabesine synthesis can be inherited by both root and shoot even though only one third of the chromosomes present in the cells of these organs are obtained from the *glauca* parent. The quantities of anabesine actually produced, however, are still smaller than those found in the parent species.

Hyoscyamine. Hyoscyamine and its racemic form atropine occur in a number of species of *Datura*, *Hyoscyamus*, *Scopolia*, *Atropa*, and *Duboisia* (43). The last named is of considerable interest, for at least two species, *myoporoides* and *hopwoodii*, contain nicotine and probably nornicotine in addition to hyoscyamine and hyoscyne. The phylogenetic significance of this observation (46-49) is not clear: a biochemical common denominator between the pyridine and tropane alkaloids, while offering intriguing possibilities for speculation, remains to be established.

Historically, the possibility of localized synthesis of the tropane alkaloids has long been tacitly assumed. The early experiments of Laurent (77), Daniel (19-21), Strasburger (132), and Lindemuth (79) were principally concerned with fruitless attempts to prove that mydriatic alkaloids, supposedly synthesized in the

leaves of *Atropa*, *Datura*, etc., could be translocated across a graft union to accumulate in the tissues of a nonalkaloid-producing rootstock such as potato or tomato. In 1941 Krajevoj and Nechaev (72) used human subjects and the electrocardiograph to demonstrate the presence of atropine (hyoscyamine) in tomato fruits that had grown on *Datura* rootstocks. Kerkis and Pigulevskaya (65) detected atropine in tomato scions on *Datura*. Hieke (45) grew various graft combinations of tomato with *Atropa* and *Datura* and came to the conclusion that mydriatically active alkaloids appear only when either *Atropa* or *Datura* serves as the root system in graftage and that the alkaloid is not confined to the tissues in which it is produced but can be transported across graft unions to accumulate in the tissues of the nonalkaloid-producing species. This view has been confirmed partially with respect to *Atropa* by Cromwell (18) and with respect to *Datura* by Peacock, Leyerle, and Dawson (99). The latter investigation relied upon direct isolation of crystalline hyoscyamine and preparation of derivatives in order to demonstrate alkaloid accumulation in tomato scions grafted to *Datura* and its complete absence in *Datura* scions grafted to tomato. These authors interpreted their data to mean that hyoscyamine is produced only in the root of *Datura* and probably also of *Atropa*. The possibility of transport of intermediates from root to leaf followed by final combination in the leaf was eliminated by the facts that the sap which bled from the surfaces of excised *Datura* stems gave a positive Vitali reaction and a strong mydriatic test. Both Hieke (45) and the latter authors obtained mydriatically active extracts from tobacco leaves grafted to *Datura* and *Atropa*. Likewise, mydriatically active extracts were prepared from the spent culture fluids of excised *Datura* roots in sterile culture (99). James (54) has provided data which can be used to support the above hypothesis of root localization of hyoscyamine synthesis. *Atropa* scions were grafted to tomato rootstocks and the alkaloid-free *Atropa* leaves that subsequently developed were employed in culture experiments. Hyoscyamine accumulation in these leaves under a variety of conditions could not be demonstrated. Lowman and Kelly (82a) demonstrated appreciable accumulation of hyoscyamine in the leaves and fruits of tomato scions grown on *Datura* rootstocks. As much as 13.00 mg. of alkaloid were found in a pound of fresh fruit. Such factors as variety of tomato, cultural conditions, and stage of maturity were involved in the concentration of alkaloid found in any given sample. The results are consistent with the view that hyoscyamine is produced in the root system of *Datura*.

James has, however, attempted to show that the failure of graft leaves to accumulate alkaloids is a consequence of an actual loss of hyoscyamine synthetic capacity as a result of grafting (54,57). According to this view intact leaves of *A. belladonna* possess some small but real ability to synthesize hyoscyamine, and the total alkaloid content of the aerial parts of the plant is therefore to be ascribed partly to synthesis in leaves. Two lines of evidence are advanced in favor of this interpretation. Detached leaves of intact plants of *Atropa* were cultured in replicate batches on solutions containing ammonium sulfate or arginine. Subsequent assays by a modified Vitali-Morin colorimetric reaction (55) indicated amounts of total alkaloids significantly greater statistically in these batches as compared with those receiving only water. Secondly, differences in the intensity of histochemical precipitation reactions with Bouchardat's alkaloid reagent (io-

dine dissolved in potassium iodide solution) were obtained between the apical meristems of root and stem in germinating seedlings of *Atropa* and also of *Datura*.

The difficulty of securing initially uniform replicate batches of leaves with respect to weight and alkaloid content for use in culture experiments has already been emphasized. It need only be added that in such cases analysis of variance and realization of statistical odds do not guarantee correct identification of the source of variance, or, in cases involving such slight increases and so many unidentified variables, factual significance based upon statistical theory. The applicability of histochemical observations to questions involving synthetic loci has also been discussed earlier and needs no further comment here. No entirely convincing evidence has been adduced to support the contention that graft leaves have lost the ability to synthesize hyoscyamine. In view of preceding considerations their failure to synthesize the alkaloid greatly decreases the probability of such synthesis in leaves of intact plants.

Hyoscyne. Recently, Hills, Trautner, and Rodwell (47) have presented extremely interesting evidence regarding the origin of hyoscyne. In their preliminary experiments tomato scions were grafted to defoliated stocks of *Duboisia myoporoides*. Although these two species are members of the same family (*Solanaceae*) one is a herbaceous vine under the usual conditions of cultivation, and the other is a rapidly growing tree. After several weeks of growth upon *Duboisia* rootstocks, however, the tomato scions contained 1% of alkaloids on a dry weight basis. Hyoscyne was obtained from the scion material as the crystalline picrate in good yield. The reciprocal graft combination could not be obtained. It was hence impossible to discover whether hyoscyne is also synthesized in the stems and leaves of *Duboisia*. In view of the relatively high concentration of the alkaloid in the tissues of the tomato scions, however, it would appear that most of the hyoscyne is produced in the *Duboisia* root and transported to the stems and leaves of the growing tree. This evidence would seem to place hyoscyne in the same category as hyoscyamine and nicotine, that is, representing essentially a product of root metabolic activity.

Lupine Alkaloids. Moshkov and Smirnova (92,124) have studied the accumulation of alkaloids in reciprocal grafts of sweet lupines with the bitter variety and with peas. Sweet lupines increased their alkaloid content when grown upon bitter lupine rootstocks, and the latter acquired a slightly lower content when grafted to the former. Notable is the fact that pea scions grafted to lupine rootstocks contained appreciable amounts of the alkaloids. The intact pea plants contained none. This may mean that the biogenesis of

the lupinane alkaloids is also a function of root metabolism. Future developments will be awaited with interest.

C. ALKALOIDS PRODUCED IN LEAVES

Anabasine. The evidence has been discussed in a previous section for the view that this alkaloid is produced in shoots as well as in roots. While it seems probable that excised leaves of *Nicotiana glauca* should be found to synthesize anabasine during culture in water, existing reports of such synthesis must be discounted until definitive evidence is available (28). Such leaves represent, nevertheless, potentially valuable experimental objects for the investigator of biosynthesis. When methods have been developed for measuring rates of anabasine synthesis in these leaves free from the complications of translocation, their use may become more valuable experimentally than the use of sterile cultures of excised *N. glauca* roots. It may be noted, in anticipation of a later section, that the most valuable leaves for this purpose will be obtained from *N. glauca* scions grown on nonalkaloid rootstocks such as tomato, since these leaves will contain only anabasine and not nicotine and/or nornicotine as do the leaves of intact *glauca* plants. Alkaloid production in the leaves of *N. glauca* is of interest from another point of view. It follows from the fact of its occurrence that the synthesis of alkaloids is not an inherently unique property of root tissues.

Nornicotine. Nornicotine is a major alkaloid in certain wild species of *Nicotiana* and in certain of the milder flue-cured tobaccos of the southern United States (37). The mechanism of its production in the plant is of interest in view of the apparent fact that as selection for progressively lower nicotine content in these tobaccos has been accomplished the content of nornicotine has increased accordingly. This would imply some type of substitution of one alkaloid for the other in the synthetic process. Grafting experiments revealed the unexpected fact that tomato scions grown on *Nicotiana glutinosa* rootstocks contained not nornicotine but nicotine alone. *N. glutinosa* scions from tomato rootstocks were essentially alkaloid free (29). In view of the fact that the shoots of *N. glutinosa* normally contain a mixture of nornicotine and nicotine, principally the former, it would seem that nornicotine arises by a secondary modification of preformed nicotine and further that this modification

occurs only in the shoot at the expense of nicotine brought up from the root. Thus, nornicotine in contrast to anabasine is primarily a product of leaf metabolism and completes the trio of possibilities, (1) synthesis only in root, (2) synthesis only in shoot, and (3) synthesis in both root and shoot.

The utility of grafted materials for alkaloid research reaches a new high level in the case of nornicotine. Theoretically, it would seem that, if nicotine is converted to nornicotine only in the shoot, it should be possible, by culturing excised shoots of plants previously grafted to a nonalkaloid rootstock, to obtain an all-or-none response to various experimental treatments. This has been accomplished in the case of *Nicotiana glutinosa*. Scions were grafted to and grown upon tomato stocks. The resulting alkaloid-free twigs were excised and cultured in solutions of nicotine salts. Toward the end of the culture period when proteolysis was evidently occurring at a rapid rate (141), nicotine was converted to nornicotine at an average rate of 0.10 gram per 100 grams dry twig weight per day (unpublished data). If such results are confirmed by direct isolation of the reaction product, little doubt can obscure the drawing of conclusions, since it is not necessary to take into account the wide variations in twig alkaloid content that occur at the outset of experiments employing nongrafted material.

A similar picture of nornicotine production has been obtained in the case of *Nicotiana glauca* (29). Certain strains of this species when grafted to tomato stocks contain no nornicotine, whereas in the intact plant an appreciable amount of the alkaloid may occur. When tomato or tobacco scions are grafted to rootstocks of *N. glauca* strains, however, nornicotine is replaced by nicotine (29,30). If *N. glauca* scions are grafted to rootstocks possessing a much higher nicotine-producing potential than *glauca* roots (e.g., *N. tabacum*) the nornicotine content of the scions may very considerably exceed the normal levels of concentration.

Unfortunately, nornicotine and anabasine are difficult to identify and to determine quantitatively in mixtures. They both distill slowly with steam, precipitate with silicotungstic acid, and form crystalline picrates. Since they are secondary amines in contrast to nicotine they are converted into relatively nonvolatile *N*-nitroso derivatives with nitrous acid and into acetylated derivatives with acetic anhydride or acetyl chloride. Furthermore, identification of the picric acid salts is greatly complicated by the ready formation

of eutectics and eutectic mixtures, the physical properties of which (e.g., melting point and solubility in water) vary extensively according to the conditions under which crystallization occurs (29). For instance, as may be seen from Table III the presence of nornicotine up to the extent of 30% in a mixture of this alkaloid with anabasine will depress the melting point of anabasine picrate only five or six degrees Centigrade. Several instances are recorded in the literature where melting points that are low by this order of magnitude have been advanced as evidence in support of the occurrence of anabasine alone (126).

TABLE III
EFFECT OF VARIOUS PROPORTIONS OF NORNICOTINE ON MELTING POINT
OF ANABASINE DIPICRATE (29)

Nornicotine pic- rate, %.....	100	95	90	80	75	70	60	50	40	20	0
Melting pt. (ap- prox.), °C.....	192	190	188	189	191	192	196	201	205	211	213

As a consequence of these difficulties, there have appeared, especially in the Russian literature (35,36,71,75,119-123,126,134,151) a large number of investigations which purport to show that the ability of *Nicotiana glauca* to synthesize anabasine is not only quantitatively increased by grafting with *N. tabacum* but also predominates over the nicotine-forming capacities of the tobacco stocks and scions to the virtual exclusion of nicotine accumulation in such graft combinations. It was held, for instance, that the absence of nicotine in tobacco scions grown upon *N. glauca* rootstocks is a result of actual suppression of nicotine-synthetic capacities of tobacco leaves under the specific influence of the stock. Conversely, the exaggerated anabasine content of *N. glauca* scions on tobacco roots was considered to indicate the complete dominance of anabasine over nicotine synthesis plus an intensifying action of the normally high-alkaloid-containing tobacco rootstock on the actual rates of anabasine synthesis in *N. glauca* scions. The genetic improbabilities surrounding such an interpretation have been outlined earlier. Suffice it to say that quantitative analyses of such graft combinations for nornicotine in addition to anabasine and nicotine would undoubtedly have revealed the true nature of alkaloid accumulation patterns to be essentially as outlined

in the paragraph above (29,30). It may be noted that the demonstration of independence of anabesine and nicotine synthetic mechanisms destroys one of the heretofore most convincing arguments of the proponents of LaMarckian inheritance.

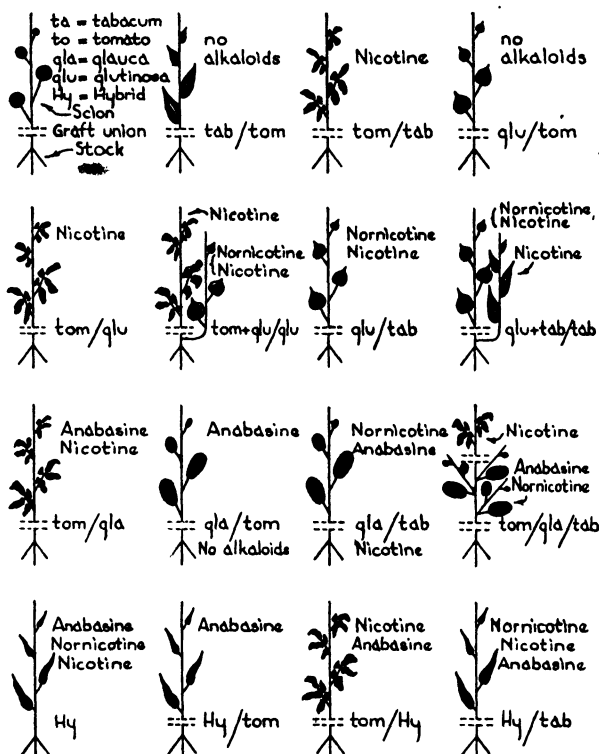


Fig. 1. Diagrammatic representation of alkaloid accumulation patterns in grafts of *Nicotiana tabacum*, *N. glauca*, *N. glutinosa*, and *N. tabacum* \times *N. glauca* with tomato (29). The various alkaloids are listed opposite each plant organ in the relative order of abundance for that graft combination.

Because of the presence of both nornicotine- and anabesine-producing mechanisms in the leaves of *Nicotiana glauca* it seems unlikely that these organs would provide advantageous experimental material for the study of biosynthesis of nornicotine at least.

Finally, the unity of alkaloid production phenomena in the genus *Nicotiana* is again demonstrated by the fact that in the two species which have been studied to date nornicotine is formed only in the leaves and only at the expense of nicotine obtained from the roots. A general picture of alkaloid accumulation patterns in the three *Nicotiana* species and the one hybrid discussed here is given in Figure 1.

D. ALKALOIDS OF UNCERTAIN ORIGIN

There have been a number of investigations which for a variety of reasons have not yet resulted in complete solution of the problem of localizing the alkaloid synthetic mechanisms in question. These are outlined briefly below. Blank (8) has already called attention to the importance which the root seems to play in many of these cases, but in the light of earlier discussions it would seem advisable to reserve judgment on this score until more complete evidence is at hand.

Cinchona Alkaloids. As a consequence of the commercial importance of these alkaloids, particularly quinine, a great amount of study has been directed toward determining their origins within the various organs and tissues of the *Cinchona* tree. Lotsy reported the results of his extensive histochemical examinations of all parts of the tree (81,82). The customary reagent, iodine in potassium iodide solution, was employed, and as a result of these observations and of the outcome of some culture experiments Lotsy drew the conclusion that alkaloids are formed in the *Cinchona* leaves during the day and are translocated downward during the night where they accumulate preferentially in the bark of trunk and root. Van Leersum (137,138) criticized Lotsy's conclusions and reported the results of his own experiments which failed to confirm those of Lotsy. In particular, an influence of light and darkness upon the alkaloid content of *Cinchona* leaves could not be detected. The most damaging evidence, however, was van Leersum's discovery that alkaloids do not accumulate in branch bark above the point of removal of a ring of bark and vascular tissues (*i.e.*, the phloem) that lie external to the cambium. Still further evidence against the possibility of alkaloid synthesis primarily in leaves was van Leersum's finding that, if a branch is tip-grafted with another species of *Cinchona* and the leaves of the former removed, the alkaloid content of the bark of the former will remain unchanged. Finally, it would seem that, if alkaloids are translocated downward from leaves and deposited in the bark, scions

of high-quinine-yielding species when grafted to the roots of a low-quinine-yielding species should bring about an enrichment with respect to quinine in the tissues of the rootstock. Fortunately, a vast accumulation of data and practical experience is available in connection with this point, for the commercial Ledger strains of *Cinchona* have long been grown in Java as scions grafted to low-grade but vigorously growing rootstocks of the species *succirubra* (91). The general conclusion seems to be that if there is any effect of grafting at all it is to increase to a small extent the quinine content of the stock and the cinchonidine content of the scion for a short distance on either side of the graft union (a full account of this problem will appear elsewhere). The possibility that leaves represent the principal site of alkaloid synthesis in *Cinchona* has thus apparently been eliminated (compare, however, Weevers, 143,144).

As early as 1869 Howard suggested that alkaloids are probably synthesized *in situ* in the cells of the bark beginning from the cambium outward (52,53). Broughton (9) agreed with this interpretation on the basis of the purely localized changes in alkaloid distribution that occur in renewed bark. There seems to have been little further experimentation in this direction, however, until relatively recent times. The author has isolated small amounts of quinine as the tartrate (confirmed by fluorescence tests), cinchonidine, and appreciable quantities of the so-called amorphous alkaloids from large volumes of the sap that bleeds from the wood (*i.e.*, the xylem) of decapitated *Cinchona* saplings. The alkaloid content of the raw sap as estimated by intensity of fluorescence quenchable by sodium chloride and by silicotungstic acid turbidity (76) was appreciably lower when collected from high-topped trunks than it was when obtained from low stumps. This latter fact may have indicated deposition in the stem tissues, presumably cortex and bark, during passage of the transpiration stream through the trunk and thus suggests the root as at least partial source of the normal alkaloid complement of the aerial tissues. The tentative results of experiments still in progress show, however, that synthesis *in situ* may account for the origin of a considerable portion of the bark alkaloids of *Cinchona*, in agreement with the earlier suggestions of Howard and Broughton. Reciprocal grafts have been obtained between *Cinchona* species and a species of the closely related but essentially alkaloid-poor genus *Ladenbergia*. Qualitative tests by the fluorimetric method, including the quenching reaction with

chlorides, for quinine and quinidine indicate an appreciable concentration of such alkaloids in *Cinchona* scions grown on *Ladenbergia* roots. The data from the completed experiments and the data concerning the occurrence of alkaloids in the xylem sap will be published elsewhere.

Berberine. Cromwell (16) has studied the distribution of berberine in *Berberis darwinii*. On the basis of the fact that this alkaloid seems to accumulate principally in the roots and root barks, Blank (8) has suggested that berberine may be produced in the roots of this species. Further data will be needed to substantiate the suggestion. Considerable importance may be attached to the solution of this problem in view of the fact that berberine represents a major group of alkaloids, the isoquinoline group, which has not thus far received extensive consideration from the viewpoint of biogenesis.

Hordenine. Raoul (103-105) has investigated the distribution of the alkaloidal amine hordenine in the germinating barley seed. According to Raoul the resting seed contains no hordenine. During germination, either in the field or during the malting process, the amine appears only in the rootlets at the points of intense cell division. The concentrations at these points increase for a time and thereafter decrease to zero. The conclusion supported by Blank (8) is that the base is synthesized only in the root tips. For reasons outlined earlier the results of this study can be accepted as indicating nothing more than accumulation in root tips. The extent to which translocation occurred prior to such accumulation and the degree of localization of synthesis in any part of the germinating seedling are unknown and cannot be inferred from the available evidence.

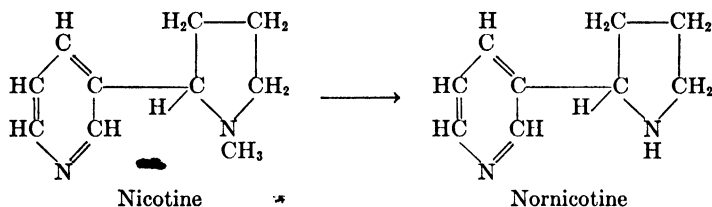
III. Mechanisms of Synthesis

While the mechanism for total synthesis is not known for a single alkaloid, the identification of sites of synthesis in the plant body has provided the experimenter for the first time with suitable material for speeding the progress of such discoveries. A few examples of recent developments in this field may be outlined herein.

A. NORNICOTINE AND METHYL GROUP TRANSFERS

It has been shown earlier that nornicotine arises from nicotine in the leaves of certain species of *Nicotiana* (29,30). The over-all mechanism involved appears to be an exchange of a methyl group for

a hydrogen atom (Scheme I). There are at least two apparent means by which this could be accomplished. One is an oxidative hydrolysis (37) yielding nornicotine and formaldehyde. The more probable mechanism is a transmethylation involving nicotine as a methyl donor, some other molecule(s) as acceptor, and an enzyme system. The



SCHEME I

evidence for the second alternative is largely indirect. First, the capacity for nornicotine synthesis from nicotine is inherited: the demethylation cannot occur if suitable genes are not present in the leaf cell nuclei (29,30,32,126). Secondly, preliminary data suggest that the rate of demethylation is considerably more rapid in leaves undergoing excessive proteolysis and in which downward translocation is blocked by excision (unpublished data). Indeed, the rate of demethylation in intact leaves is so slow that nornicotine-producing species nearly always contain greater or lesser amounts of as yet unchanged nicotine in their leaves (126). Whatever element of the system is responsible for the sluggishness of the reaction in these cases, the inherent capacity of such leaves to transform nicotine into nornicotine has not been curtailed, for leaves grown on plants grafted to the high-nicotine-producing *Nicotiana tabacum* are capable of considerably greater rates of demethylation (29). It would thus seem that the limiting factor in most cases is the supply of the hypothetical methyl acceptor rather than a lowered activity of an enzyme system. Plants have long been known to contain a wide variety of methylated substances including choline, betaine, stachydrine, trigonelline, and the purines. Du Vigneaud and associates (34) have shown that methyl groups are actively metabolized in the animal body and that enzymic transmethylations provide the pathways for the necessary methyl group transfers. It would seem, therefore, that the subject of transmethylations in plants is ripe for study and should yield rich rewards to the interested experimenter. In connection with alkaloid biogenesis, the problems of the origin and possible exchange of methoxyl and

methylenedioxy groups should also be investigated and may provide some interesting parallels (9).

B. NICOTINE AND ORIGIN OF THE PYRIDINE RING

Chemically, the quinoline, isoquinoline, phenanthridine, quinuclidine, tropane, and lupinane ring structures may all be regarded as representing various modifications of the pyridine ring. The only major alkaloid group not represented in this proposed category of pyridine derivatives is that based on the indole nucleus. From the biosynthetic point of view it is also an interesting fact that alkaloids tend to occur in any given genus or species of plant in one or at most a few homologous series based upon modifications of a common ring structure. Pyridine forms the basic nucleus of the tobacco alkaloids with serial substitutions in the 3 position by derivatives of pyrrole, pyrrolidine, pyridine, and piperidine (37). The quinuclidine nucleus with variously modified quinoline or even indole (44,66) rings attached through a secondary carbinol bridge make up the framework of the *Cinchona* alkaloids. Many other examples may be given. If the above assumption is justified, however, the great majority of alkaloid molecules may be regarded as representing essentially a series of modifications and/or combinations of pyridine and indole rings (149).

There is increasing evidence to indicate that the simplest pyridine derivative in nature, nicotinic acid (niacin), can be synthesized in a variety of organisms from one of the most universally distributed indole derivatives, the amino acid tryptophan, or at least that the synthetic mechanisms for these two substances may follow in part a common pathway (74,113). Tatum, Bonner, and Beadle (133) have shown that tryptophan is synthesized in *Neurospora* according to the reaction sequence, anthranilic acid \rightarrow indole (+ serine) \rightarrow tryptophan. The steps by which the latter may participate in nicotinic acid synthesis are as yet unknown. That the synthesis may actually be fairly complicated would seem to follow from a current study of the inheritance of the niacin content of corn grain (unpublished). In this case, at least, the quantitative production of nicotinic acid is determined primarily by a considerable number of genes of small individual effects, with some complicating factors. Less is known about the inheritance of alkaloids, but it may be inferred from existing data (29,69-71,125,126) to be under a type of quantitative control perhaps similar to that for niacin in corn.

If a definite biosynthetic relationship is established between tryptophan and nicotinic acid, considerable interest will attend any effort to find whether or not a similar transformation may account for the origin not only of the pyridine alkaloids but also those of the quino-line, isoquinoline, quinuclidine, phenanthridine, tropane, lupinane, and indole groups and of trigonelline as well (see above). Julian (62) has suggested a connection between tryptophan and the biosynthesis of physostigmine in the Calabar bean, and Winterstein and Trier (149) have discussed the possibility of the origin of other nitrogen heterocycles in the plant cell from tryptophan.

Unfortunately, the only experimental data that might have related nicotinic acid (and, hence, by inference also tryptophan) to alkaloid synthesis have been obtained with plant organs incapable of producing alkaloids. Klein and Linser (67), Gorter (39), Dawson (24), and Ciferri and Pratesi (15,102) have attempted to identify the precursors of the pyridine ring of nicotine by feeding excised tobacco leaves with a variety of substance including glutamic acid, proline, ornithine, and nicotinic acid according to the suggestions of Trier (149). Klein and Linser reported increases in the nicotine content of the leaves when proline and ornithine were supplied. Gorter could not confirm these results. Dawson reported especially large increases in the nicotine content of tobacco leaf blades when the excised shoots to which they were attached were fed nicotinic acid. Ciferri and Pratesi report that nicotinic acid was effective only when a mineral nutrient solution containing inorganic nitrogen was supplied to the leafy shoots. Dawson has since employed initially nicotine-free tobacco leaves from plants grafted to tomato rootstocks and has failed repeatedly to obtain a synthesis of nicotine from proline and nicotinic acid (unpublished data). In view of these results it is therefore necessary to ascribe the data of Klein and Linser, Dawson, and Ciferri and Pratesi to something other than alkaloid synthesis. If a synthetic relationship were to exist between nicotinic acid and nicotine (and there is no evidence that it does) it will be detected through the use of excised root cultures and not by the use of excised tobacco leaves.

C. HYOSCYAMINE AND INTERMEDIARY ROLE OF ARGININE

Considerable attention has been given to the identification of intermediates in hyoscyamine synthesis as a result of the stimulus provided by Robinson's suggestions of possible mechanisms and his reali-

zation of these mechanisms by model syntheses *in vitro* (87,108,109, 110). According to Robinson the amino acids lysine and arginine constitute the probable starting points for the biosynthesis of the solanaceous alkaloids. Lysine is thought to give rise to piperidine and even pyridine ring structures, while arginine is thought to undergo hydrolysis to yield ornithine, which in turn is converted to the tropane and pyrrolidine rings. Theoretical objections to this and similar views of biogenesis have been outlined earlier in the present essay from the standpoints of biology and biochemistry. Therefore, discussion is limited herein to the results of those investigations which have sought to test the validity of Robinson's hypotheses by actual experimentation at the *in vivo* level.

Cromwell (17) grew *Atropa belladonna* plants in sand culture and noted increases in the alkaloid content (determined by micro-Kjeldahl measurements of the total nitrogen of alkaloidal extracts) of the leaves when various sources of nitrogen and carbon were presented simultaneously to the roots. Etiolated plants supplied in the dark with potassium nitrate and glucose also contained higher concentrations of alkaloid. Experiments with detached leaves cultured under a variety of conditions gave increases in alkaloid concentration in some cases but not in others. The results within a given treatment were also inconsistent. The possibility of drawing a definite conclusion regarding the question whether an actual synthesis of alkaloid occurred in any case is unfortunately removed by the fact that the investigator did not record the changes in dry mass of his plants and plant parts that must have occurred parallel to the observed changes in alkaloid concentration. Cromwell (18) attempted a second investigation of alkaloid synthesis in *Atropa* with the aid of somewhat more acceptable methods of analysis (*i.e.*, titration). Although the results of grafting experiments with tomato are reported to correspond with the results recently obtained by Hieke (45), no satisfactory quantitative data are presented to support this claim. Alkaloids were, however, detected in appreciable amounts in the sap that bled from decapitated *Atropa* stumps. A novel method of feeding relatively large volumes of solutions containing various hypothetical intermediates was developed, and, although the details of application and manipulation are not clearly given, small but consistent increases in alkaloid content of root and leaf tissues were obtained with arginine plus glucose, formamol plus glucose, hexamine plus glucose, and with

putrescine with or without glucose. Since putrescine is a metabolite of arginine, the relationship of this substance to the observed increases in tissue alkaloid concentration was pursued in a succeeding publication (18). Small but apparently consistent increases in diamine oxidase activity were obtained using suitable extracts of *Atropa* roots, shoots, and etiolated shoots when putrescine was added. Simultaneous tests for ammonia and aldehyde that would be expected to result from the oxidation of putrescine appeared to follow expectations. If putrescine were actually to serve as an intermediate in hyoscyamine synthesis, it was reasoned, the amine might occur in *Atropa* tissues in detectable amounts at least during periods of most active alkaloid synthesis. Cromwell reports the isolation of putrescine from the leaves and upper stems of *Atropa belladonna* and also from *Datura stramonium* from material gathered in August but not in May or June. In the light of these findings he suggests that arginine may exist in equilibrium with proteins on the one hand and that it may be converted successively to ornithine, putrescine, and succindialdehyde or amino aldehyde on the other hand. The latter are regarded in the light of Robinson's speculations as prime intermediates for alkaloid synthesis.

James has continued the experimental approach of Cromwell (54-58) and reported the apparent synthesis of alkaloids in detached belladonna leaves fed with ammonium sulfate plus sucrose and also with arginine. The suggestion was made that arginine may be converted to ornithine and the latter in turn oxidized by an α -amino acid oxidase to α -keto- δ -aminovalerianic acid. If this reaction sequence actually occurs in belladonna tissues it should be possible according to James to detect the enzymes that catalyze each of the component steps. The presence of arginase was reported in root and shoot extracts of not only belladonna but also tobacco and tomato (54). Arginase activity was detected in *Datura* shoots and in the shoots of belladonna grafted to tomato stocks. The latter were essentially alkaloid free, and it may be noted that, while James uses the presence of arginase in intact belladonna plants to support his hypothesis of the mechanism of hyoscyamine synthesis, its presence in the alkaloid-free leaves of grafted belladonna can with equal logic be used as an argument against his view that these leaves have lost their capacity for alkaloid synthesis (see page 225). When arginine feeding was employed the tissues of the leaves exhibited symptoms of ammonia toxicity. Tests

with belladonna root sap (leaf extracts were not employed) indicated the presence of urease. James, therefore, concludes that the nitrogen of the tropane alkaloids is probably derived from the δ -amino group of arginine and ornithine and that the α -amino nitrogen of other amino acids including proline is not available for the synthesis.

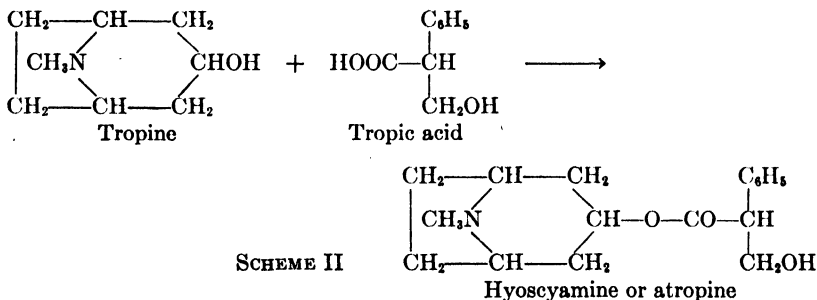
Underlying the results of all these investigations is the assumption that *Atropa* and *Datura* leaves are capable of carrying out the total synthesis of hyoscyamine beginning with such simple materials as ammonia and sugar. In an earlier discussion of this problem the validity of this assumption has been seriously questioned. There are, moreover, certain additional objections to the assumptions implicit in such experiments as have been described above. In the first place, ammonia, arginine, and ornithine would be expected to occur in virtually all plant tissues (as would also the enzymes by means of which these substances are geared to protein metabolism and to amino acid degradation) as a consequence of the operation of the arginine cycle of Krebs and Henseleit (73) and Srb and Horowitz (130). Therefore, the detection of the enzymes arginase, urease, and even diamine oxidase can have nothing more than a mechanistically remote, indirect, and entirely nonspecific connection to alkaloid synthesis in any plant. The same may be said for an earlier suggested relationship of tryptophan to the pyridine alkaloids. All plants would be expected to contain some tryptophan and some arginine in their proteins as well as to possess enzymic mechanisms for their degradation. If a connection is to be established between an amino acid of practically universal occurrence and the synthesis of an alkaloid of highly restricted occurrence in nature, it will be necessary to look for evidence at the point where the metabolism of the amino acid enters upon a unique pathway such as, for instance, in the suggested conversion of arginine not to ornithine and α -keto- δ -aminovalerianic acid but to some substance which may represent the first step in the specific series of reactions leading only to the synthesis of the tropane ring. Under these circumstances experiments designed to obtain accessory evidence by testing for enzyme activities will not deal with those enzymes responsible for the normal metabolism of a commonly occurring amino acid but will attempt to cope instead with the problem of identifying enzymes which facilitate such reactions as ring closures, dehydrogenations, etc. Finally, in connection with the isolation of putrescine from *Atropa* and *Datura* shoots (18), it may be noted that a

variety of amines are normally encountered in the aerial parts of plants, particularly in the upper portions of stems and their inflorescences (27,68). If the synthesis of putrescine is actually based upon the action of an enzyme on ornithine obtained from arginine, the evidence of Cromwell may be interpreted in a number of ways with equal facility. The simplest explanation would seem to be that the formation of putrescine in small amounts represents an alternative pathway for arginine metabolism in these two species, that such formation is in part terminal, and that, therefore, the syntheses of putrescine and hyoscyamine are entirely independent.

It would appear that future advances in the field of hyoscyamine biosynthesis will come most readily from the use of organ cultures of established alkaloid-synthesizing capacities. *Datura* roots have been grown successfully in sterile culture, and there is no *a priori* reason to presume that belladonna roots cannot be grown in a similar fashion (99,146,147).

D. TROPINE AND A PROBLEM OF ESTERIFICATION

It would seem reasonable to suppose that a more profitable approach to the problem of alkaloid biosynthesis may lie in the direction of the relatively obvious terminal reactions such as the methylation of the tobacco alkaloids and the esterification of the tropane alkaloids. Hills and associates (49) in Australia have performed an experiment of great interest in this connection (see Scheme II). A scion of *Nicotiana tabacum* was grafted to a rootstock of *Duboisia myoporoides*.



The scion grew well and flowered. Whereas an intact tobacco plant of the same variety employed as a control contained 0.2% nicotine in its leaves on a dry weight basis, the grafted tobacco also contained 0.2% of steam-volatile alkaloids calculated as nicotine. Two crystal-

line picrates were obtained from the nonvolatile alkaloidal fractions. A small amount of hyoscyne picrate comprised one of the fractions. The other and larger fraction proved to be tropine picrate! At first thought it is not easy to explain the fact that unesterified tropine should occur in tobacco leaves grafted to *Duboisia* roots since it is not present when *Duboisia* roots are attached to their own shoots or to tomato scions (46-48). A further puzzling fact is that tropine should apparently accumulate but not scopine, since both bases are present in esterified form as hyoscyamine and hyoscyne, respectively, in the intact plant. Hills, Trautner, and Rodwell (49) have suggested that the basic parts of the alkaloid molecules, tropine and scopine, are formed in the roots and later transferred to the leaves of the intact *Duboisia* tree where esterification with a suitable acid takes place. These authors suggest that this process may be prevented in the tobacco graft through the lack either of a suitable acid or of an esterifying enzyme or both. An alternative explanation would be the possibility that tobacco leaves contain an enzyme capable of hydrolyzing hyoscyamine but not hyoscyne. This possibility might easily be tested experimentally by feeding the alkaloid on the one hand and its acidic and basic components on the other to tobacco leaves of the same strain as that used in the Australian experiments. The second possibility would seem the most likely in the light of the detection of fully assembled hyoscyamine in the ascending transpiration stream of *Atropa* and of *Datura* (18,99).

Further important contributions are to be expected from the investigation of alkaloid formation in the *Duboisia* genus.

IV. Related *in Vivo* Mechanisms

A. ALKALOID SYNTHESIS AND PROTEIN METABOLISM

Perhaps more than any other group of substances excepting, of course, the amino acids, the alkaloids have been associated in the physiological and biochemical literature with one phase or another of protein metabolism. Henry has given an excellent statement and discussion of this tendency in the introduction to his book on the plant alkaloids (43). It is necessary here to emphasize the conclusion drawn by Henry that the technique of experimentation in this field is difficult and that the results obtained are frequently open to more than one interpretation. Indeed, a careful inspection of such results beginning with the papers of Pictet, who originated the idea of alka-

loid-protein relationships (100), and continuing to the more recent studies of Cromwell on berberine (16), Annett on morphine (2,3), and Weevers on the *Cinchona* alkaloids (143) reveals a complete lack of anything more than purely circumstantial evidence, which evidence is open to a rather wide variety of other interpretations. As Henry has remarked, attempts to relate alkaloid formation to protein metabolism in plants are unusual in view of the relatively small number of species in the plant kingdom that contain alkaloids.

Annett (2,3) employed a number of variables including sodium nitrate fertilization to show that the alkaloid content of the latex of the opium poppy (*Papaver somniferum*) remains essentially constant even though the growth of the plant and the production of latex in the plant were greatly increased in some cases. The relative constancy of the morphine content of this latex and the fact that the alkaloid was not used in the ripening of the seed led Annett to suggest that morphine is an end product of metabolism and excretory in nature. The experiments of Cromwell (17,18) on hyoscyamine formation in *Atropa belladonna* have been summarized in a preceding section. Their relevancy to questions of the relationships of alkaloids to protein metabolism is considerably in doubt in view of the fact that Cromwell did not in a single instance demonstrate an increase in absolute alkaloid content of his plants and leaves. Nevertheless, the conclusion was drawn that the alkaloids are dependent for their synthesis upon certain by-products of protein metabolism, especially ornithine and putrescine, and that these latter substances in turn are involved in condensation reactions with substances derived from carbohydrate metabolism to produce the finished alkaloid molecules. Weevers (143,144) has discussed the general problem of the relationship of the purines and alkaloids to protein metabolism and has stated his view, based upon experiments with the *Cinchona* alkaloids, that the latter substances are probably formed by dissimilative processes in the plant. Mothes (93) was not able to detect a relationship between nicotine content of tobacco leaves and either protein synthesis or protein decomposition, but such research as that of Sabalitschka and Jungermann (111,112) has been cited repeatedly as providing evidence for the view that alkaloids are synthesized from the reserve proteins of germinating seeds (43,143). While Vickery (139) failed to isolate any simple soluble substance from mature tobacco seed which might reasonably be assumed to act as the precursor of the nicotine that ap-

pears during germination, the possibility that such substances may exist has not been eliminated.

The most recent advocate of the association between alkaloid synthesis and protein metabolism is James (54,57). Although certain of the basic arguments upon which his stand is based have been seriously questioned in earlier portions of the present essay, James's evidence is so skillfully organized and presented that a brief summary is recorded herein as follows:

(1) Alkaloids are absent in the embryo and endosperm of the dormant seeds of *Atropa belladonna* and *Datura stramonium*. They appear very early during germination and according to histochemical tests seem to accumulate most abundantly in the apical meristems of the seedling root and shoot. During growth, as in the case of nicotine in tobacco, the amount of alkaloid in the aerial portions continues to increase with the highest concentrations again occurring in the meristems both lateral and apical, in the young cortical cells, pith cells, and in the phellogen. In older leaves an actual loss may occur during senescence. The above facts, according to James, are in agreement with the view that alkaloids are synthesized in the young and actively growing cells of the plant body regardless of their location and that they are broken down during senescence. The synthesis of alkaloids would thus appear to occur parallel to that of the proteins in the development of the individual plant with the conditions that favor one process also favoring the other.

(2) Alkaloid synthesis can apparently be intensified by excessive nitrogen fertilization, the control of available potassium and phosphorus supplies, and by inducing proteolysis (*i.e.*, by keeping detached leaves in the dark in distilled water). Feeding ammonium sulfate plus sucrose to excised leaves in the dark is also claimed to result in increased alkaloid content of the leaves. Since leaves under such circumstances appear not to synthesize protein it would then seem that alkaloid formation may as readily be associated with protein degradation as with protein synthesis.

(3) The common denominator between protein synthesis and protein degradation is probably an abnormally high tissue concentration of amino acids, and it matters not whether they are of primary or secondary origin. Not all amino acids are considered to be equally available for alkaloid synthesis: glycine, alanine, valine, leucine, glutamic acid, histidine, and proline were ineffective in feeding experi-

ments, while arginine and ornithine gave small increases in young but not in old leaves.

(4) The presence of arginase and urease in belladonna tissues was demonstrated as accessory evidence for the postulated mechanism of synthesis via arginine.

It may be noted that an almost identical marshalling of evidence can be obtained for the origin of nicotine in tobacco (10-15,23-25,31, 67,100,102), notwithstanding the fact that more recent evidence has shown this picture of nicotine biogenesis to be entirely circumstantial and erroneous.

In general, the principal argument for schemes such as this is the correspondence in time of maximum rates of alkaloid accumulation with events within the plant, such as growth or autolysis, which are presumed to indicate maximum rates of protein formation or degradation. It is at once the strength and the fatal weakness of such schemes that they are never based upon actual measurements of the prevailing rates of over-all protein metabolism, nor, indeed, are they always based upon actual quantitative measurements of the rates of alkaloid synthesis *per se*! Vickery and associates (140) in their study of the chemical changes that occur during the growth of the tobacco plant have provided the only relatively complete set of data at present available for comparing rates of alkaloid accumulation with simultaneous rates of net protein production. Unfortunately, for the purpose at hand, this study was confined to the aerial organs of the tobacco plant, and it is therefore not possible to compare the above variables for the root organs in which the nicotine was actually synthesized. It is notable, however, that no intelligible relationship emerged between nicotine and protein accumulation in leaves and stems. The literature of plant physiology since the days of Pfeffer has leaned strongly to the assumption that appreciable rates of protein metabolism occur only during certain critical events in ontogenetic development such as seed germination, meristem activity, and senescence. If the concept of continuous protein turnover in cells as developed by Rittenberg, Schoenheimer, and Keston (106) and extended to plants by Vickery *et al.* (142) is correct, it would appear that efforts to correlate the occurrence of such phenomena as alkaloid synthesis and protein metabolism by the methods employed thus far cannot be expected to avail much. The author of this essay has earlier and elsewhere (31,32) withdrawn his claims for a relationship between

nicotine synthesis in tobacco leaves and protein metabolism via amino acids such as proline (23,24). It seems probable, in view of these and earlier considerations regarding the ability of excised leaves to synthesize hyoscyamine, that the very similar picture of protein-alkaloid relationships developed for belladonna must either be supported by an *experimentum crucis*, perhaps involving isotopic nitrogen, or likewise be regarded as having no more than a coincidental basis in fact.

B. ALKALOIDS AND GENERAL PLANT PHYSIOLOGY

Alkaloids in many species are characteristically associated either as esters or as salts with certain organic acids. For instance, tropic acid, atropic, benzoic, tiglic, and other acids are esterified with basic alcohols such as tropine, nortropine, or scopine to form the belladonna and related alkaloids. In *Cinchona* barks quinine, quinidine, cinchonine, cinchonidine, and many others are associated with a variety of more or less characteristic acids including quinic and cinchotannic (9,33). It has been suggested from time to time since Justus von Liebig advanced his "mineral theory" (78) that alkaloids may serve as substitutes for inorganic cations of soil origin to insure the neutralization of these organic acids for which there seems to be no further use in the plant (9). It would be extremely difficult to devise experiments calculated to prove beyond a doubt that alkaloids actually perform such a function in plant physiology. Undoubtedly the alkaloid molecules, their ions, and hydrated forms exist in plant tissues in complex homogeneous or heterogeneous equilibrium with all other charged particles that occur simultaneously in these same tissues. Organic acids of many types must be presumed to participate in such equilibria. No complete attempts to trace the role of an alkaloid in such a system have been made, although Broughton (9) failed to find a correlation between the alkaloid content of *Cinchona* barks and their principal ash constituents.

The most complete study of the relationships of an alkaloid to the physiology of the plant which produces it has been made by Mothes (93). Although many of his results are obscured by the fact, then unknown to him, that nicotine synthesis is confined to the root system of the tobacco plant, Mothes was nevertheless able to demonstrate the following salient features of tobacco physiology: (1) nicotine accumulates in the plant whenever there is growth; (2) nicotine metabolism is very stable and is not easily influenced; (3) nicotine accumu-

lates in the presence or absence of light and at the expense of reserve foodstuffs if necessary; (4) the rate of alkaloid production is not in accord with the rate of growth of the leaves; (5) nicotine cannot act as a nitrogen source for protein synthesis during nitrogen starvation; and (6) during the catabolic changes that occur in senescent leaves, the secondary degradation products of nicotine are of no appreciable significance in the physiology of the plant. Dawson (25) has confirmed the inability of nicotine to serve as a nitrogen source for protein synthesis in excised tobacco shoots during culture.

While nicotine is known to be translocated in large amounts from root to shoot, ~~it~~ has not been observed to move downward (basipetally) anywhere in the plant body. Such translocation would presumably occur in the phloem tissues of stem and leaf and could be used as evidence indicative of the functioning of nicotine in the movement of organic or inorganic anions from one part of the plant body to another. Dawson could not detect such movement in appropriately grafted plants (27,28,29), and Mothes reported similar failure in his study of intact tobacco plants (93).

It has been observed (22,25) that nicotine salts fed to excised tobacco shoots considerably increase the water content of the shoot tissues as well as the rate at which these shoots absorb water to replace transpiration losses. These effects were not specific, however, and could be duplicated with glutamic acid or nicotinic acid (22,23). Wiley, in a continuation of this line of study, compared the rates of water absorption by preconditioned (131) potato disks from solutions containing nicotine hydrochloride and potassium chloride in equimolar concentrations (148). No significant differences were observed. Attempts to attribute some special survival value to the presence of alkaloids in plants have frequently been made, but these suggestions have not seemed to deserve serious biochemical consideration.

Frankenburg (37) has suggested that alkaloids may participate as catalytic agents in plant tissues even though they may not enter directly into the chemical conversions which they regulate. The highest importance must be attached to this possibility, since it is conceivable that the catalysis may be either negative or positive in a broad sense (38). The formal relationship of the pyridine alkaloids to the pyridine moiety of the di- and triphosphopyridine nucleotides of the dehydrogenases has been pointed out earlier (31,37), and the possibility of participation of these and other alkaloids as hydrogen donor

and acceptors in oxidation-reduction systems should provide a fertile field for investigation. Dawson has called attention in a preliminary way to the results of some experiments in which nicotine was found to increase the rate of nitrate absorption and possibly also of nitrate reduction in the roots of tobacco plants growing in sand culture (32). The study of this phenomenon is continuing, but attention may be called here to two of its more obvious implications regarding the design of experiments. First, the physiological significance of any alkaloid will be most reliably indicated by observing its action upon the species of plant in which it is produced. Secondly, only those cells or tissues within the plant body which are actually capable of synthesizing the alkaloid in question can be expected to yield the answer to the problem of its biochemical activity.

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CERTAIN ASPECTS OF THE MICRO-BIOLOGICAL DEGRADATION OF CELLULOSE*

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I. Introduction

Cellulose is considered to be the most abundant organic compound existing in nature. It is the skeletal framework of all higher plants, whether in the free state as it exists in the seed hairs of cotton, or in a union with other organic compounds as is the case in wood where it exists combined with lignin. However, whether this union is physical or chemical still remains to be established. Considering the abundance of this compound in nature, it is no wonder then that man has attempted, rather successfully, to put it to use in the form of rayon, cellulose plastics, cellophane, and as various derivatives. Nor can we lose sight of the tremendous consumption of cellulose in the form of clothing, rope, wood, and paper.

While man attempts to find use for this compound which exists so ubiquitously in nature, nature, too, has provided for the continuous destruction of cellulose—almost entirely through the action of micro-organisms. Of the organisms capable of decomposing cellulose the following groups are included: anaerobic bacteria, aerobic bacteria, filamentous fungi, many types of higher fungi, certain actinomycetes, various protozoa, certain types of insects, and a variety of other invertebrate animals, including worms and snails.

II. Structure of Cellulose

A. CHAIN STRUCTURE

Before entering into any discussion involving cellulose, it will be attempted to clarify the subject under consideration as much as possible. Generally speaking, the constitution of cellulose is well established. However, in view of certain suggested modifications of the classical theory of cellulose structure a short discussion of its constitution is presented here in order to afford a contrast with the recent concept.

Indication that its structure is that of a hexose anhydride is afforded by its empirical formula, which has been shown to be $C_6H_{10}O_5$. The most uniform cellulose is obtained from the seed hairs of cotton. Since the seed hair contains 99.8% cellulose it is the purest form of the compound and is thus termed "true cellulose." From the study of this form of cellulose many data on its chemical constitution have been obtained.

Of prime importance in establishing the structure of cellulose was

the necessity to demonstrate the relationship between glucose and cellulose. Complete acid hydrolysis of cellulose gives an almost quantitative yield of glucose, indicating at the same time the qualitative relationship that exists between the two compounds. As early as 1819 Braconnot (10) discovered the qualitative relationship between cellulose and glucose, the complete acid hydrolysis of cellulose giving rise to glucose. Monier-Williams (50), over a century later was able to show this relationship to be nearly quantitative. Employing cold 72% sulfuric acid, he dissolved the cotton and completed the degradation to glucose by diluting the solution with much water to an acidity of less than 1% and boiling the solution for fifteen hours. On the basis of the optical rotation and copper reduction he was able to show the presence of about 94.5% of glucose.

The qualitative and quantitative relationship between cellulose and glucose being established, it remained to determine the linkage between the glucose units constituting the cellulose molecule. Of importance in this effort was the observation of the presence of three alcoholic hydroxyl groups for each $C_6H_{10}O_5$ unit. This was ascertained on the basis of nitration and acetylation of cellulose. This was also indicated by the formation of a trisodium derivative by the action of metallic sodium on cellulose in liquid ammonia, with the liberation of hydrogen. The studies of Scherer and Hussey (76) corroborated the above finding. They found that three sodium atoms entered each $C_6H_{10}O_5$ unit. Although the presence of three alcoholic hydroxyl groups in the glucose unit of the cellulose molecule was established, it remained to determine the positions occupied by these groups. With the knowledge of the structure of glucose itself and of the presence of these reactive groups, too, it would be simpler to conceive of the linkage existing between the glucose units to form the cellulose molecule, if knowledge of the positions of the three reactive groups in the glucose unit were available.

Methylation studies with cellulose have brought to light evidence to clarify this point. Cellulose was methylated and hydrolyzed with hydrochloric acid and methanol, giving rise to methyl 2,3,6-trimethyl- β -D-glucoside. On hydrolysis of this compound 2,3,6-trimethylglucose resulted (38). This observation has had much influence on the formulation of the constitution of cellulose. The identification of 2,3,6-trimethylglucose would settle the position of the three hydroxyl groups in the glucose unit of the cellulose molecule as being in posi-

tions 2, 3, and 6. Also of importance in clarifying this point was the finding by Haworth and Machemer (28) of 2,3,4,6-tetramethylglucose in the hydrolyzate. Hence, in cellulose, these three hydroxyl groups are not involved in the glycosidic linkages. This elimination leaves either of the hydroxyl groups in the 4 and 5 positions for combination with the potential aldehyde in the 1 position of another glucose residue. On the assumption that position 5 is involved in the internal ring, as in the case with glucose, the glucose units must be joined in the cellulose molecule through positions 1 and 4 since these are the only available positions. Also in view of the fact that the additional hydroxyl group methylated in 2,3,4,6-tetramethylglucose is at position 4 it must be surmised that the linkage between the glucose units occurs at the 4 position and not the 5 position. It would appear, then, that in practically the entire cellulose molecule, positions 1, 4, and 5 are "blocked" and cannot be subject to esterification or etherification. The above findings regarding the linkage between the glucose units signify that one of the end groups in the cellulose molecule contains an additional alcoholic hydroxyl group in position 4, on the basis of the formation of 2,3,4,6-tetramethylglucose.

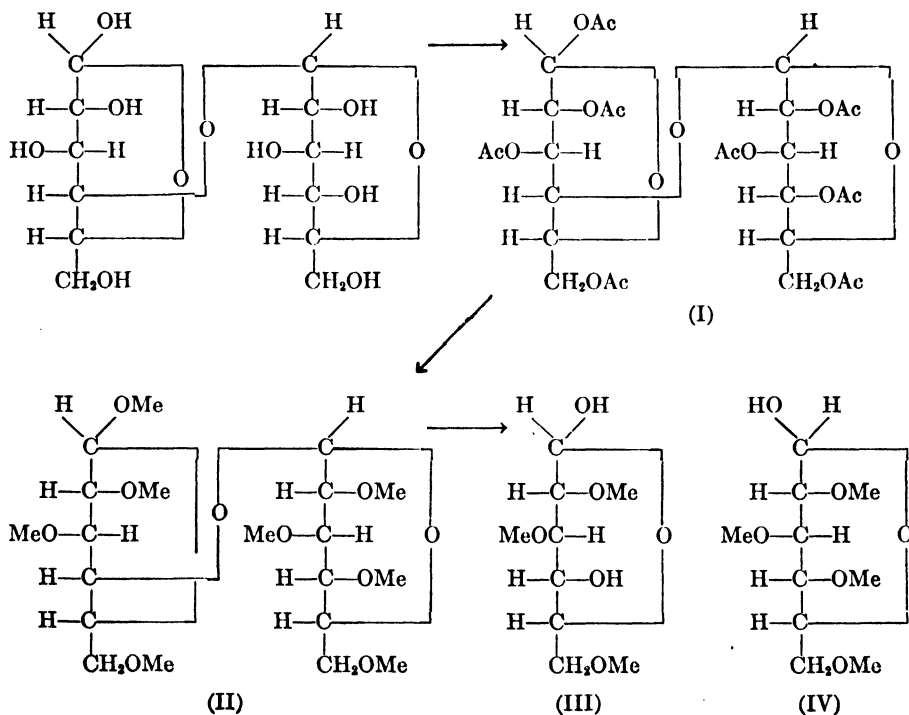
Although the alcoholic hydroxyl groups in the cellulose molecule on the basis of the above evidence were located at carbons 2, 3, and 6 of the glucose residues, the mode of linkage between the glucose units was left unsettled. It might be possible that the glycosidic bond possessed either the α or β configuration and it might be attached to either carbon 4 or 5 in the adjacent glucose unit. Some doubt, therefore, existed as to whether the glucose units possessed a 1,5- or 1,4-cyclic structure.

A method employed to clarify this uncertainty was the characterization of breakdown products obtained from cellulose intermediate in complexity between cellulose and glucose. In this attempt the acetolysis of cellulose has served to contribute much to the elucidation of the constitution of cellulose. A crystalline octaacetate of cellobiose was prepared on treating cellulose with acetic anhydride and sulfuric acid (20,78). The formation of two moles of glucose was observed in the acid hydrolysis of the free sugar, obtained by the saponification with sodium ethylate of the octaacetate derivative. Similarly, glucose resulted from the hydrolysis of cellobiose with almond emulsin. This latter finding characterizes cellobiose as a β -glucoside.

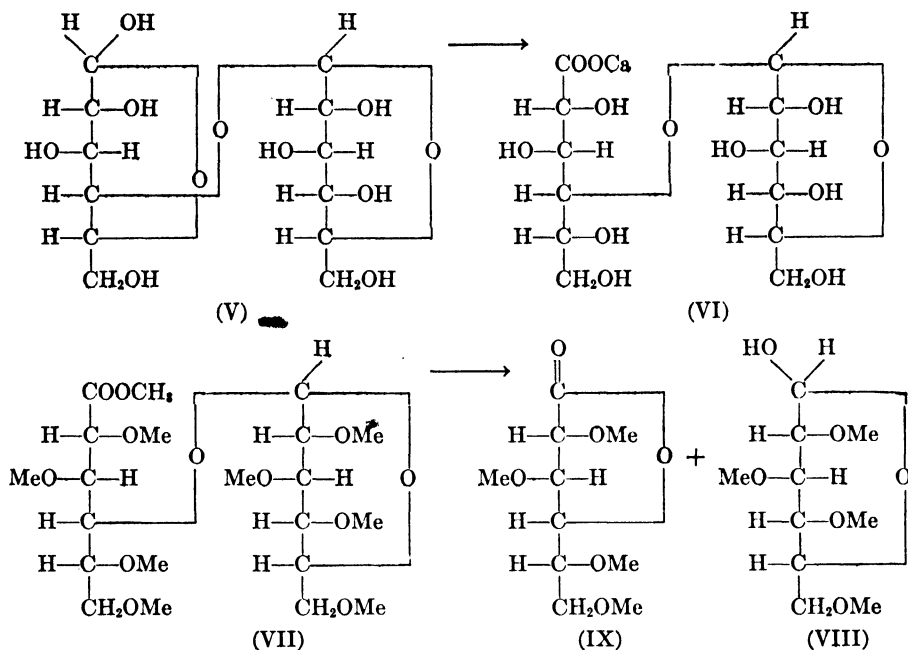
Studies on the structure of cellobiose were performed by Haworth

and collaborators (26). The configuration of this disaccharide was established through a series of reactions. By controlled hydrolysis it was obtained in considerable quantities, indicating that the sugar is preformed in the cellulose molecule (87). It is a reducing sugar hydrolyzed by acid or by almond emulsin into two moles of glucose.

Crystalline cellobiose octaacetate (I) was simultaneously deacetylated and methylated with dimethyl sulfate and alkali to form a crystalline methyl heptamethylcellobioside (II), hydrolysis of which resulted in crystalline 2,3,6-trimethylglucose (III) and 2,3,4,6-tetramethylglucose (IV) (Scheme I). These findings would limit the disaccharide linkage to carbon 4 or 5. Evidence for selection of carbon 4 was given by Haworth, Long, and Plant (27). Cellobiose (V) was oxidized to calcium cellobionate (VI), which on complete methylation produced methyl octamethylcellobionate (VII). Hydrolysis of this compound gave rise to 2,3,4,6-tetramethylglucose (VIII) and 2,3,5,6-



SCHEME I



SCHEME II

tetramethylglucosyl lactone (IX) (Scheme II). These observations strongly support the concept that carbon 4 of the reducing half of the molecule is joined through the oxygen bridge to carbon 1 of the non-reducing half of the molecule in the original cellobiose.

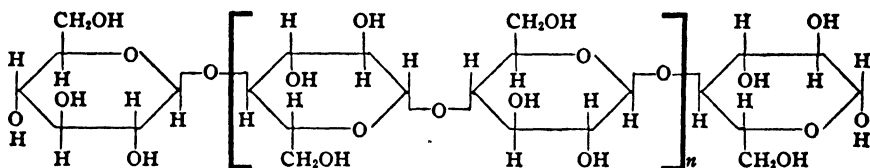
It would be assumed, then, that the sugar cellobiose (linkages of which seem to be preformed in cellulose) is 4- β -D-glucopyranosyl-D-glucopyranose. The structure of cellobiose was further confirmed by the synthesis of octamethylcellobiose by the interaction of 2,3,4,6-tetramethylglucosyl chloride and methyl 2,3,6-trimethyl- β -D-glucoside (21). This, too, is evidence of a β -glycosidic linkage.

During the acetolysis of cellulose, acetates of oligosaccharides, as well as cellobiose octaacetate, are formed. "Cellotriose," a trisaccharide, was reported as being obtained as a product by Bertrand and Benoist in 1923 (6). It was shown that "cellotriose" has a structure which follows the same pattern as does cellobiose and only β linkages appear to be present (22). Similarly, a tetraose and a pentaose have been obtained during the acetolysis of cellulose. All these

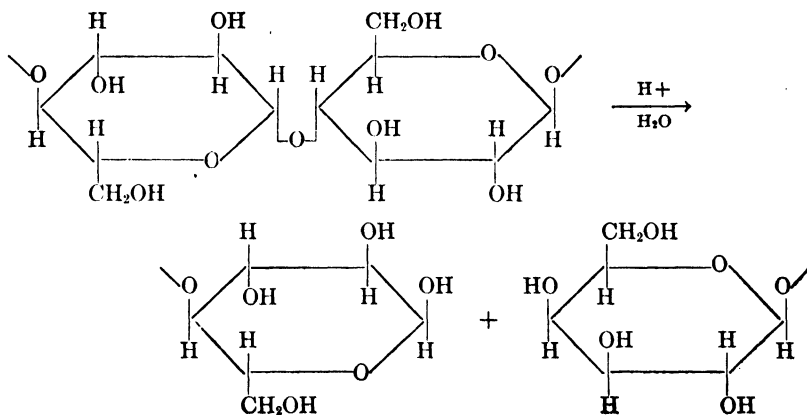
sugars appear to represent chains of glucose units possessing similar structures and configurations. Too, they contain glucopyranose groupings which are linked together as β -glucosides, through positions 1 and 4 of adjacent glucose units.

B. THE TOLLENS-HILLER-PACSU CONCEPT

Based on the afore-mentioned data, cellulose is generally accepted today to be made up of β -D-glucopyranose residues united by 1,4-glycosidic linkages. Also, it is generally assumed that hydrogen bonds, $O-H \cdots O$, are present. The individual chains are thought to be held together in a lateral direction mainly by these bonds:



On the strength of recent analytical work on cellulose, and previous considerations of Kleinert, Hingst, and Simmler (44), Pacsu and Hiller (34) extended Tollens' concept of an acetal structure for cellulose and conclude that the present picture is not complete in that it does not satisfactorily account for the chemical behavior of this compound. Heretofore, the formation of glucose during the acid-catalyzed hy-



SCHEME III

hydrolysis has been interpreted as being due to a cleavage of the 1,4-glycosidic bonds of a chain molecule. The result of the action of acid would be a diminution of the average degree of polymerization. The product of the partial hydrolysis of cellulose is termed "hydrocellulose," the hydrolysis proceeding as in Scheme III.

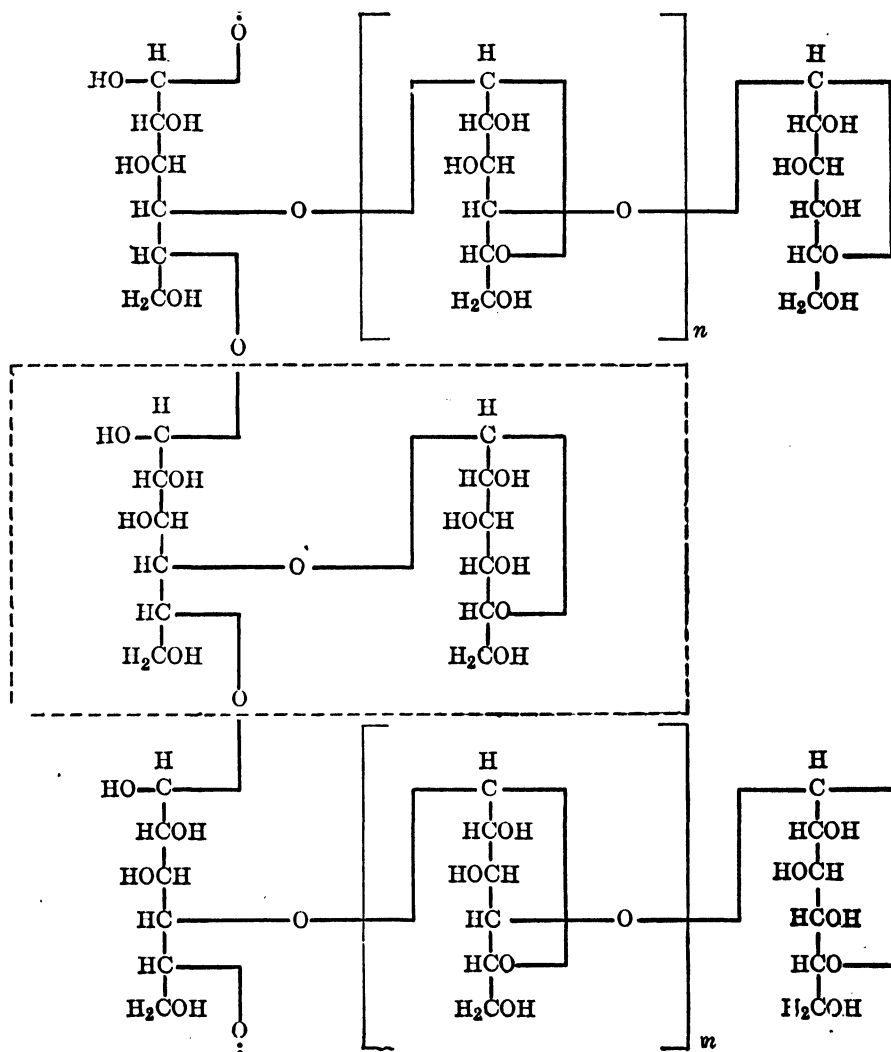
It would be assumed that every time a hydrolytic cleavage occurs, a new reducing group is formed. Applying earlier methods used for determining reducing groups, a "hydrocellulose" should show a greater reducing power than the original substance from which it was prepared. This has not been found to be the case. A method customarily employed has been the procedure originally developed by Schwalbe (77) and termed the "copper number" method. In principle it consists of the reduction in alkaline media of cupric ions, thus causing the formation of a red precipitate of cuprous oxide, and the oxidation of the cellulose. The amount of cuprous oxide is then measured by standard methods. The inadequacies of this method are generally known. Under the conditions employed it serves to give erratic results.

Another often-applied method is the oxidation of cellulose by hypiodite solution. This method, too, has been frequently criticized. It has been found that the oxidizing agent itself was unstable. Using any of these methods accuracy and completeness cannot be expected.

However, with a group estimation method based on the use of dilute potassium permanganate in acid medium, results were obtained which were contrary to the above expectations. Hiller and Pacsu were surprised to find that an *initial* rapid acid treatment (mild in nature) of cellulose decreased its reducing power rather than increased it. It is interesting that the reducing power lost by the cellulose was reported to have been found in the acid solution used for degrading the material. This was considered to indicate that during this *initial* rapid effect of dilute acid on cellulose, bonds other than normal glycosidic bonds are broken. These findings led the authors to believe that, besides normal 1,4-glycosidic bonds, cellulose contains another type of linkage which is extremely sensitive to acid-catalyzed hydrolysis. They conclude that the cellulose fiber is made up of a chain of molecules which range in size from long chains down to a few which are probably glucose or cellobiose. The primary-chain molecules with their terminal open-chain units form still larger, secondary-chain molecules by means of hemiacetal linkages, reminiscent of those of

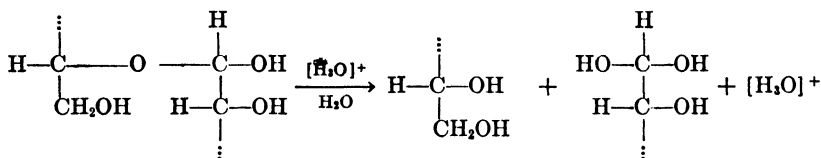
Tollens which involve open-chain molecules of glucose and cellobiose. Part of such a structure is shown below in Scheme IV.

This formula is regarded by Hiller and Pacsu as adequately representing the chemical structure of a portion of the cellulose molecule,



SCHEME IV

two molecular chains being held together by a cellobiose molecule. They surmise that the *initial* action of acids is not hydrolysis of the 1,4-glycosidic bonds, but rather hydrolysis of the hemiacetal linkages between the hydrated aldehyde groups and any of the alcoholic groups of the glucose unit in the main chain. Of course, while it is thought that splitting of 1,4 linkages will take place under more drastic conditions, the properties of "hydrocellulose" do not arise from this attack. The initial action of acids is supposed to be the hydrolysis of the hemiacetal linkages and the cleavage of a number of hydrogen bonds which normally hold the fiber together. A split of the hemiacetal linkage would occur as is illustrated in Scheme V.



SCHEME V

Since the newly created small molecules are freed and leave the fiber, upon being washed, they do not give rise to new reducing groups; the reducing power of the "hydrocellulose" formed becomes less than that of the original material from which it was prepared on acid hydrolysis. The authors, however, did not mention whether or not prolonged *enzymic* hydrolysis might give analogous results.

C. PHYSICAL EVIDENCE REGARDING CELLULOSE CONSTITUTION

The present-day generally accepted concept of the chemical constitution of cellulose pictures it as being made up of long chains of β -D-glucopyranose residues united by 1,4-glycosidic linkages. It would appear, however, that the structure of cellulose is not completely represented as such. X-ray analyses have helped to clarify this. Thus x-ray analysis has definitely shown that cellulose is of a crystalline nature, and the individual chain molecules are thought to be parallel to each other. In turn, they are stabilized laterally by secondary valences or by hydrogen bonds between opposing hydroxyl groups. Meyer and Mark (49) have reported that the cellulose micelles are composed of a definite number of long-chain molecules

held together by lateral secondary cohesion forces. A regular crystalline structure was assumed. It is thought that in such a manner chain bundles are formed, such bundles being conceived as representing hypothetical submicroscopic units of varying length, but of fixed breadth and thickness. These are termed crystallites or micelles. However, the cellulose structure is now considered to be made up of both crystallized *and* amorphous areas.

The results of x-ray analysis tend to support the afore-mentioned molecular chain structure of cellulose. Thus, the length of the identity period of the elementary cell in the direction of the fiber axis (*b* axis, 10.2 Å) is identical with the length of two pyranose residues which are combined with each other as in cellobiose and connected with other glucose units at their ends.

The crystallinity of cellulose was first established by means of x-rays by Nishikawa and Ono (52). Contributions by Polányi (69) and Polányi and Herzog (31) furnished the fundamental data according to which the cellulose fiber was regarded as representing a crystal lattice in which rows of crystals are arranged essentially in a position parallel to the fiber axis. At this time Polányi made the first attempt to determine the unit or basic cell, *i.e.*, the smallest unit which still possesses the geometric characteristics of the whole crystalline lattice. It appears that the lattice on which the cellulose crystal is built may be ascribed to the monoclinic system with dimensions of the basic cell expressed in angstrom units:

<i>a</i> (horizontal).....	8.45 Å
<i>b</i> (vertical, representing the length of the basic cell, parallel to the fiber axis).....	10.2 Å
<i>c</i> (forming the angle with <i>a</i>).....	7.9 Å
β	84°

X-ray analysis also shows a considerable portion of a fibrous system of cellulose not to be crystalline. This portion of the system is ascribed to the amorphous part of the structure. The cellulose fiber is, therefore, regarded as a two-phase system, consisting of the same chemical material combined in two different types of physical aggregation. There is a crystalline region in which the chain molecules are thought to be arranged strictly parallel, and an amorphous region in which the chains are less parallel. The term micelle is used to designate the crystalline regions. The chain bundles are thought to

be separated by intermicellar spaces of amorphous regions and these spaces account for the penetrability of the fibrous system by liquids, dyestuffs, etc.

D. SIMILARITY OF WOOD CELLULOSE AND "TRUE" CELLULOSE

The nature of cellulose just discussed holds for "true" cellulose. Wood contains cellulose as a principal constituent. During the process of enzymic decay of wood, by the action of wood-destroying molds, this constituent is degraded. Much interest has been directed toward the mechanism of the destruction of the cellulose fraction of wood during such decay. Part of the following presentation is concerned with the problem. Before embarking on such a discussion, however, it is advisable that we try to clarify certain aspects of the subject under consideration. Would, for instance, the nature of wood cellulose be identical with that of "true" cellulose? The properties of "true" cellulose have been attributed to wood cellulose too. It would seem that the two celluloses are identical. Like cotton cellulose, wood cellulose is considered to be made up of glucose groups with β -glucosidic linkages connecting positions 1 and 4.

Several facts attest to the identity of the two celluloses. An indication of the similarity is apparent from the fact that both are capable of acetolysis. The work of Heuser and Boedeker (33) supports the hypothesis that wood cellulose and cotton cellulose are chemically identical. Wood cellulose which had been purified by means of 17% NaOH was hydrolyzed with fuming hydrochloric acid. A control experiment was carried out with cotton cellulose. In both cases, the rate of hydrolysis to glucose was very nearly the same. A triacetate which then was converted into methyl glucoside by means of methanol and hydrochloric acid was obtained by Heuser and Aiyar (32) from spruce pulp. Analogous observations were made using cotton cellulose. Additional evidence supporting the identity of the two celluloses was afforded by the work of Wise and Russell (90). Working with cotton cellulose and spruce cellulose, they obtained identical yields of cellobiose octaacetate after acetolysis of both. Similar nitrates and acetates have been found from both types of samples.

These data are but a few which support the contention that "true" cellulose and wood cellulose are identical. It is justified, then, to think of wood cellulose as possessing identical chemical properties with those ascribed to cotton cellulose.

III. General Considerations of the Enzymic Degradation of Cellulose

A. CONCEPT OF PRELIMINARY HYDROLYSIS

Depending on the type of microorganism originating the degradation of cellulose, a variety of end products are obtained. Regardless of the organism causing the breakdown of cellulose, it is generally surmised that an exoenzyme brings about hydrolysis of the insoluble* cellulose molecule to give rise to soluble glucose, which upon entering the cell is dissimilated intercellularly.

The nature of this preliminary hydrolytic action is not fully understood. If it were to be assumed that the cellulose molecule was represented by simple chains of glucose units linked through 1,4- β -glucosidic bonds, the hydrolytic action might consist of chopping off terminal glucose units, with a consequent shortening of the chain. However, considering the probability of cross linkages holding the chains together, it is conceivable that two distinct hydrolytic processes are taking place: (1) one breaking the cross linkages that hold the chains together and (2) a second type of hydrolysis effecting the splitting off of glucose units. The reason for such an assumption is that insoluble dextrans (chains of glucose units linked as in cellulose, the chains being shorter) can be utilized more readily than cellulose by microorganisms that attack cellulose.

B. THEORY OF OXIDATIVE DEGRADATION

Another concept regarding the primary action of microorganisms on cellulose has been advanced by Winogradsky (88). He considered the primary attack to be oxidative in nature. This theory was postulated on the basis of similarities noted by this investigator between the properties of filter paper attacked by cellulose-decomposing organisms and chemically produced oxycellulose. The suggestion was advanced that terminal carbinol groups were oxidized to aldehyde groups, and that these as well as primary alcohol groups were further oxidized to carboxyl groups. Thus, with cellulose (and wood) the increase in acidity in the culture media under the action of certain wood-destroying molds, *e.g.*, those belonging to the *Polyporus* and *Merulius* types, was considered by Lüttke (46) as one of the possi-

* So far nothing is known regarding the mechanism of enzyme action on *soluble* cellulose obtained, according to T. Lieser (*Cellulosechemie*, 18, 121, 1940, or *Kolloid-Z.*, 98, 142, 1942).

bilities due to the oxidation of potential reducing and hydroxyl groups to carboxyl groups. Actually, however, this acidity is due to the production of organic acids as metabolic products of the action of these organisms on wood. The nature of these compounds will be discussed later.

Further objections to the oxycellulose theory have been raised by Norman and Bartholomew (59) and Norman and Fuller (60). According to them the concept of oxidative conversion is not feasible because it presupposes the existence of an oxidative exoenzyme system which is highly improbable. It is not likely that an insoluble oxycellulose would be formed initially since no energy could be obtained by the cell until water-soluble fragments were formed. And, too, the fact that under no circumstances was oxycellulose positively identified as the product of the conversion of cellulose would make the concept of oxidative degradation unwarranted.

This theory being unlikely, there is reason to believe that the primary stage of the degradation of cellulose is hydrolytic in nature with the ultimate formation of reducing sugars which enter the cell and which are further dissimilated to yield a variety of end products, depending on the enzymic equipment of the organism responsible for the destruction of the cellulose.

Popoff's studies established the formation of gases as the result of the natural decomposition of cellulose (70). The gases formed were found to be methane and hydrogen. In 1886 Hoppe-Seyler (35) reported his investigations in the same field. He was the first to use Swedish filter paper as a source of cellulose in his experiments. He confirmed that hydrogen may be formed together with methane during the fermentation of cellulose. Omeliansky (62) demonstrated a quantitative transformation of cellulose, under aerobic conditions, into fatty acids consisting of acetic, butyric, and valeric acids, as well as CO₂ and methane. He gives the figures recorded in Table I for a cellulose fermentation by *Bacillus methanigenes*. The slight excess

TABLE I

Cellulose		Degradation products	
Cellulose used for experiment	2.0815 g.	Fatty acids	1.0223 g.
Residue	0.0750 g.	Carbon dioxide	0.8678 g.
Cellulose utilized	2.0065 g.	Methane	0.1372 g.
		Total	2.0273 g.

of recovered fermentation products over and above the weight of cellulose decomposed is regarded by him as within the limits of experimental error. He also studied the formation of hydrogen from cellulose by the action of *Bacillus fossilicularum*. The balance sheet in Table II is presented. In addition to the fatty acids, consisting

TABLE II

Cellulose		Degradation products	
Cellulose used for experiment	3.4743 g.	Fatty acids	2.2402 g.
Residue	0.1272 g.	Carbon dioxide	0.9722 g.
Cellulose utilized	3.3471 g.	Hydrogen	0.0138 g.
		Unidentified	3.2262 g. 0.1209 g.
		<i>Total</i>	3.3471 g.

of 1.7 moles of acetic to 1 mole of butyric acids, traces of valeric acid and of higher alcohols are formed. Utilizing the action of *Spirochaeta cytophaga* (84) on cellulose it was observed that carbon dioxide was the main metabolic product in addition to a mucilagenous material thought to be oxycellulose.

Pringsheim (73) obtained 0.213 g. formic acid and 1.15 g. acetic acid, as well as a small amount of lactic acid. Out of 42 g. of cellulose degraded, Peterson *et al.* (80) found that 21.6 g. acetic acid, 10.5 g. alcohol, and 11.0 g. carbon dioxide in addition to hydrogen were formed. Ethyl alcohol has been also found among the products of the anaerobic breakdown of cellulose by bacteria, by Khouvine (39), together with acetic and butyric acids, traces of lactic acid, carbon dioxide, and hydrogen. Working with *Bacillus cellulosa dissolvens* she was able to obtain about 8% ethyl alcohol, calculated on the cellulose fermented. Grown anaerobically, this organism could not ferment any carbohydrate but cellulose. Table III is a balance sheet for a typical fermentation by this organism as presented by Khouvine.

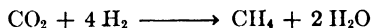
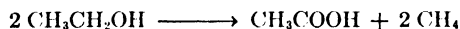
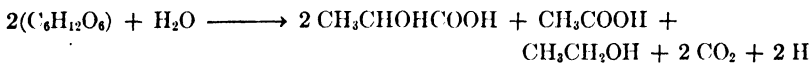
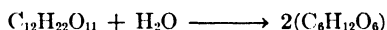
Glucose, too, has been obtained as a product of cellulose decomposition. Thus, Imshenecki (37) has demonstrated that glucose is the first breakdown product in the anaerobic breakdown of cellulose by *thermophilic* bacteria, and it accumulates in the medium to the extent of 74% of the cellulose decomposed. Alcohol, acetic, butyric, formic, and lactic acids, together with hydrogen and carbon dioxide, are then

TABLE III

CONVERSION PRODUCTS OBTAINED FOR 1.012 GRAMS DEGRADED CELLULOSE

Acetic acid.....	0.275 g.
<i>n</i> -Butyric acid.....	0.033 g.
Ethyl alcohol.....	0.082 g.
Carbon dioxide.....	0.1827 g.
Hydrogen.....	0.0085 g.
Pigment.....	0.0135 g.
<hr/>	
Unrecovered products.....	0.5947 g.
	0.4173 g.
<hr/>	
<i>Total</i>	1.0120 g.

produced from the sugar. In an attempt to explain the formation of various acids, alcohols, and gases during the anaerobic decomposition of cellulose, Lyman and Langwell (47) and Neuberg and Cohn (51) suggest the following mechanism, the reactions involved being hypothetical:



From a consideration of the above data it would appear that, in the process of degradation of cellulose by the action of microorganisms, a primary hydrolytic action takes place whereby soluble monosaccharides are produced. Depending on the organisms, the soluble carbohydrate is dissimilated in the cell, giving rise to the variety of compounds mentioned.

Evidence in support of a primary hydrolytic attack during the action of microorganisms in the destruction of cellulose has been offered. Of significance in this direction are the findings of Pringsheim (72). He postulated the existence of two hydrolytic enzyme systems, namely, cellulase and cellobiase, the former producing cellobiose from cellulose and the latter giving rise to glucose from the disaccharide. That cellobiose appeared as an intermediate was shown by Pringsheim by raising the temperature to a point at which the enzyme which breaks down cellobiose to glucose becomes inactive while the

cellulase, the enzyme which produces cellobiose from cellulose, is still active. He also reported the intermediate formation of cellobiose by the addition of antiseptics, particularly a 0.5% solution of iodoform in acetone.

The formation of copper-reducing carbohydrates as intermediate decomposition products during the microbiological destruction of cellulose was foreshadowed by Euler's experiments (18a) on the action of a juice pressed from *Merulius lacrymans* on dextrins prepared from filter paper.

IV. Biochemistry of Wood-Destroying Molds

Regardless of the type of mold causing the destruction of wood, the cellulose portion is invariably destroyed. Although decay in wood and its prevention are matters of economic importance, little is known about the mechanism of the breakdown of cellulose by the activity of these microorganisms. Perhaps one of the reasons for the lack of knowledge of the mechanism of this destruction by fungi has been the inability to isolate intermediate breakdown products, which could help to indicate the phase sequence of the formation of degradation products and, perhaps, assist in visualizing the fate of cellulose, when wood is attacked by wood-destroying fungi.

It is often stated that at least two types of wood decay are brought about by molds, namely, the brown and white rots. In the former, preferential attack is made on the carbohydrate components of wood substance, and the lignin remains in the main unaffected, the decayed residue being brown in color. In the second type lignin seems to be the main substrate of the mold and in the residue there are patches or "pockets" of a white substance said to consist of pure cellulose. Campbell (15) subdivides white rots, on chemical grounds, into three distinct groups: (a) White rots in which lignin and pentosans are attacked in the early stages and in which incidence of attack on cellulose is delayed. (b) White rots in the early stages of which both lignin and cellulose are attacked but in varying proportions. (c) White rots in which cellulose with its associated pentosans are attacked in the early stages and the lignin attack delayed. The type names are thus derived from the color of the residues after the attack.

A classification of decay types based on the chemical effects of the fungus on the host is suggested in the work of Falck and Haag (46), Wehmer (86), and Bavendamm (4). These authors favor the use of

the terms "destruction" and "corrosion," respectively, the former type to include all forms of fungal decay in which the carbohydrates are preferentially attacked, and the latter to refer to the forms in which lignin is preferentially attacked. According to Wehmer the "destruction" type is brought about by the hydrolysis of carbohydrates and in the "corrosion" type lignin is depleted by a process of oxidation.

Independent of the type of enzymic attack on wood, that is, whether the destruction results in a brown rot or white rot, cellulose seems to be a substrate of the enzyme attack in both cases.

Despite the fact that much work has been done on the study of wood decay by wood-destroying molds, comparatively few unequivocal and tangible facts are available in this field. The functional description of certain enzymes present in wood-attacking fungi appears to be somewhat haphazard (54). This, in turn, seems to be due to the uncertainty of, or difficulty in, making a clear-cut subdivision of wood-destroying fungi. As a matter of fact, Bose (9) claimed that, of the wood-rotting organisms, only about two dozen have been studied with regard to their enzyme activity.

Waksman (89) states that the mechanism of cellulose degradation by bacteria and fungi is not fully understood because intermediate breakdown substances have been but rarely isolated; therefore, a postulation of the mechanism of the destruction of cellulose is not possible. On the other hand, Wehmer (85) aptly called the wood-destroying fungi the pioneers in a process which serves to reintroduce the gigantic wood waste into the basic cycle of utilization.

The main interest of this laboratory has been, so far, centered on a consideration of the destruction of the cellulosic fraction of wood during the decay of wood by the action of this type of mold. A direct approach to the problem would consist in the isolation and identification of intermediate breakdown products. The objection to such an approach is at once obvious. Not only is the action of the organisms slow in bringing about the decay of wood, but it would also be difficult to show that any isolated breakdown product has arisen from any one of the constituents of wood.

Setting aside for the present the pertinent question as to whether cellulose is chemically combined with the lignin in wood, it was decided to study the action of wood-destroying fungi on cellulose itself, and several simple sugars. With some knowledge available as to the

terminal breakdown products of the action of this class of organisms on wood, it appeared that a correlation of these results with those obtained from a study of the breakdown of cellulose and the simple sugars could afford us a means of deducing a phase sequence for the degradation of the cellulosic constituent of wood during the decay of wood. The study of the degradation of the simple sugars by these molds has been undertaken on the assumption that a preliminary hydrolysis of cellulose occurs. Thus, it has been indicated that such a preliminary hydrolysis of cellulose to cellobiose and glucose takes place during the biological decomposition of cellulose.

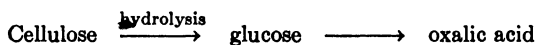
Merulius lacrymans, a brown-rot type of mold known to cause extensive decay of wood, has been shown to contain the enzymes cellulase, lichenase, β -glucosidase, and maltase. Ploetz (67) obtained pressed juice from *M. lacrymans* cultures, which, after dialysis, showed the presence of these enzymes. The fact, too, that the organism can effectively decay wood, the cellulosic fraction preferentially, and cellulose itself, to give rise to oxalic acid in either case would justify such a correlation. Similarly, *Coniophora cerebella*, a mold also causing brown rot, is capable of giving rise to oxalic acid as a terminal decomposition product of wood, cellulose, and glucose.

The one outstanding feature with regard to brown rot of wood is that, as a direct result of infection, the residue is rendered more soluble in sodium hydroxide than the original sound wood. In fact, Bray (14) has pointed out that in the case of brown rots, increase in alkali solubility serves as an index of the depletion of cellulose. It has also to be noted that acid hydrolysis has the effect of increasing the alkali solubility of wood, and this fact has led many to conclude that there must be a relationship between acid hydrolysis on the one hand and fungal decay on the other.

The literature contains many references to the acid-forming action of wood-destroying molds. Thus, *Coniophora cerebella* has been found to form acetic, formic, citric, and oxalic acids (8) as degradation products of the action of this mold on wood. Falck (19) claimed to have identified free succinic and malic acids and combined oxalic and tartaric acids as metabolic products of the same organism. The production of oxalic acid as a degradation product has also been demonstrated by Rabanus (74) as a result of the action of *Poria vaillantii* on wood, and *Merulius lacrymans*, too has given rise to oxalic acid as a metabolite. The production of acids by the action of the fungi

on wood thus points to a preliminary hydrolytic action by the organisms similar to an acid hydrolysis.

Investigations carried out in our laboratories corroborate this preliminary hydrolytic action by the molds in causing the degradation of cellulose. Thus, it has been demonstrated that *M. lacrymans*, *P. vaillantii* and *C. cerebella* give rise to appreciable amounts of oxalic acid when they attack cellulose in the form of filter paper and surgical cotton. Likewise, they are able to produce the acid by their action on glucose. Inasmuch as it has been demonstrated that the same organisms give rise to oxalic acid by their action on wood, it follows that a partial scheme of the formation of oxalic acid from the cellulosic fraction of wood follows the pattern:



Supporting evidence for the hydrolytic action of wood-destroying molds on cellulose is afforded by the results of studies of the metabolism of the mold *Lentinus lepideus*. Thus, it has been reported that this mold acts on wood (7) to give rise to methyl *p*-methoxycinnamate as a metabolic product. We have established that the organism is capable of producing the same compound via glucose. It would then appear that this fungus acts on wood, as well as on glucose, to give rise to a synthetic ester as a metabolic product; this would appear to corroborate the theory of the degradation of the cellulose of wood via an introductory hydrolytic action. According to this concept, an exoenzyme secreted by the microorganism causes the hydrolysis of the cellulose molecule. This gives rise to glucose molecules which are subsequently dissimilated by the enzymes in the cells of the organism, resulting in a variety of end products. Since it has been demonstrated that *L. lepideus* produces methyl *p*-methoxycinnamate from glucose as well as from wood, it is only through a hydrolytic split of the cellulose that glucose can arise as a primary dissimilation product. Then by further action of the organism the compound is synthesized via the soluble glucose molecule.

With the afore-mentioned background, it was considered desirable to attempt an attack on the problem of the cellulosic degradation of wood by the action of wood-destroying molds, in an effort to arrive at a comprehensive picture of the fate of cellulose. With this in mind studies have been made of the dissimilation of cellulose, its hydrolytic product, glucose, and several other simple sugars. The action of the

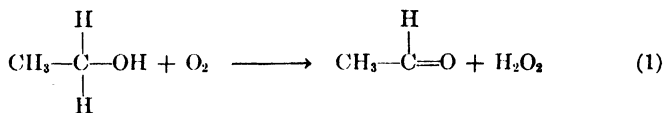
following organisms, commonly termed wood-destroying fungi, has been studied:

- | | |
|--------------------------------|------------------------------|
| 1. <i>Merulius niveus</i> | 5. <i>Merulius lacrymans</i> |
| 2. <i>Merulius tremellosus</i> | 6. <i>Poria vaillantii</i> |
| 3. <i>Merulius confluens</i> | 7. <i>Lentinus lepideus</i> |
| 4. <i>Fomes annosus</i> | 8. <i>Lenzites sepiaria</i> |
| 9. <i>Coniophora cerebella</i> | |

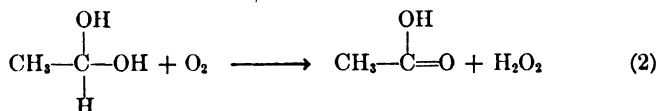
A. DISSIMILATION OF GLUCOSE BY WOOD-DESTROYING MOLDS

Organisms 1, 2, 3, and 4 belong to the group found to produce ethyl alcohol, acetaldehyde, acetic acid, and succinic acid as metabolic products, using glucose as substrate (54). Organisms 5, 6, and 9 gave rise to oxalic acid as the terminal metabolite. *L. sepiaria* produced ethyl alcohol and *L. lepideus* gave rise to methyl *p*-methoxycinnamate and traces of ethyl alcohol as metabolites (56). The formation of ethyl alcohol, acetaldehyde, acetic acid, and succinic acid by one group of organisms, on the one hand, and of oxalic acid by another group prompted the consideration of a correlation between the metabolites.

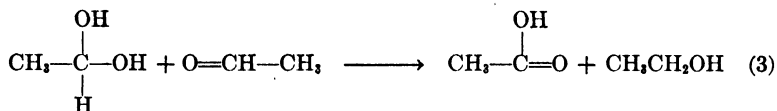
Considering the first group, it was noticed that the ethyl alcohol so formed was subject to a dehydrogenation, whereby substantial amounts of acetaldehyde and acetic acid could be formed. The isolation of acetaldehyde without interception, and of acetic acid, the accumulation of which under anaerobic conditions amounted to four times that obtained aerobically, raised the question of the origin of these compounds. Although the disappearance of ethyl alcohol, when given as a substrate, pointed to its direct dehydrogenation:



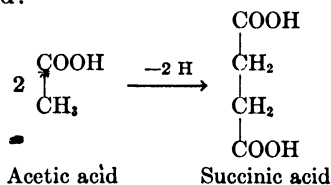
it would still be conceivable that two processes were progressing simultaneously: (a) a dehydrogenation of the acetaldehyde so formed:



or, and more so under anaerobic conditions, (b) a dismutation of two molecules of acetaldehyde:

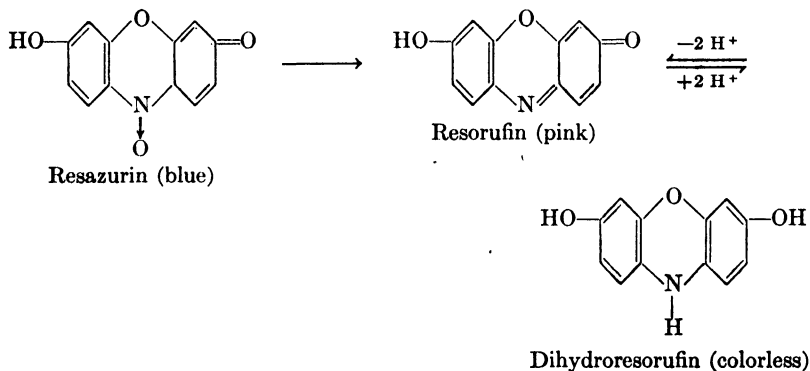


resulting in the formation of acetic acid and alcohol. The succinic acid detected could be assumed to result from a dehydrogenation of the acetic acid formed:



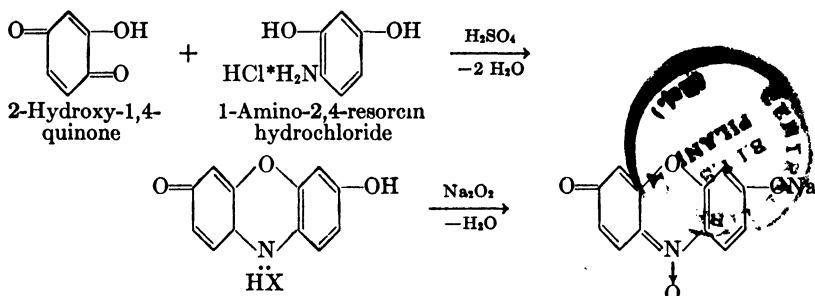
B. DEHYDROGENATION STUDIES IN THE PRESENCE OF RESAZURIN

The presence of a dehydrogenating enzyme system being indicated, it was decided to further establish its presence and to study its actions. It appeared desirable, therefore, to utilize a dye which could act as an interagent in visualizing the progress in a dehydrogenation. For this purpose resazurin, which passes through the following stages during reduction, was employed.



Inasmuch as the standard procedure for the preparation of resazurin provides for the possible admixture of some resorufin, the presence of which at the beginning of the dehydrogenation reaction could

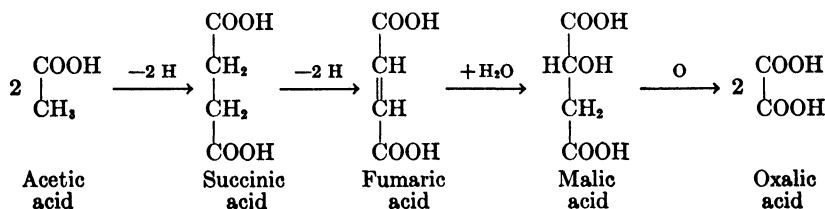
anticipate its enzymic formation, it was deemed necessary to attempt a synthesis (83) which enabled us to offset the probability of simultaneous formation of this contaminant. The synthesis is based on the following reaction:



The utilization of a strong oxidizing agent, sodium peroxide, excluded the possibility of the formation of resorufin, and supplied us with the desired uniform product.

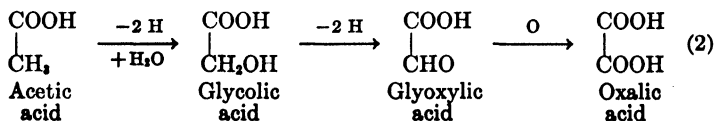
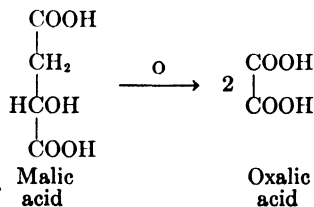
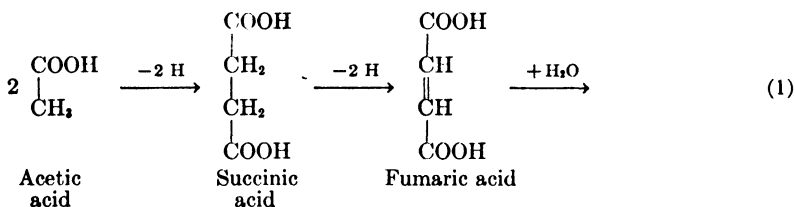
Employing resazurin as an indicator in dehydrogenation studies, the dehydrogenation of D-sorbitol and of isopropyl alcohol by the four wood-destroying molds *Merulius niveus*, *M. tremellosus*, *M. confluens*, and *Fomes annosus* was studied. The qualitative and quantitative course of the dissimilation of D-sorbitol to the ketose was established by means of the Seliwanoff test and the Munson-Walker method. The course of conversion of isopropyl alcohol was ascertained by the identification of its dehydrogenation product, acetone, as the 2,4-dinitrophenylhydrazone.

The detection of succinic acid as a metabolic product presented the problem of its possible role in the mode of formation of the oxalic acid found as the terminal metabolic product of the other group of organisms. Assuming the succinic acid to arise via a dehydrogenation of acetic acid, as indicated by the discoloration of resazurin when incorporated in acetate medium, it would be possible for oxalic acid to arise as follows:



To substantiate this mechanism the molds of the first group (page 273) were grown on a medium of sodium acetate. Resazurin, incorporated in the medium, was discolored, indicating the presence and action of a dehydrogenating enzyme. Sodium oxalate was obtained as the only metabolic product. It would appear, then, that oxalic acid was formed by the action of the molds on the acetate medium, via a preliminary dehydrogenation.

Although the consideration of the formation of oxalic acid via the pathway indicated appeared attractive and likely, it is not the only pathway conceivable for the production of the acid from acetic acid. Bearing in mind that the postulation of such phase sequence is based partly on the observation of the discoloration of the dye when included in the acetate medium, it appeared that another mechanism could also be surmised. Thus, a dehydrogenation of acetic acid could give rise to glycolic acid. Consequently, it could be assumed that the discoloration of resazurin indicated a dehydrogenation of acetic acid, resulting in the formation of succinic acid, glycolic acid, or both as transition products in the production of oxalic acid. It would be conceivable, then, that *two* routes can be followed during the formation of oxalic acid from acetic acid, namely:



In order to elucidate the mechanism of oxalic acid formation it was necessary to distinguish whether oxalic acid was formed via either or both of these pathways, and to establish which intermediary stages were passed during the genesis of the terminal acid. The solution of the first part consisted in the observation of the production or non-production of oxalic acid from succinic acid and glycolic acid, these acids being forerunners of oxalic acid in the pathways indicated. Two means for the solution of the second problem were available: a direct method consisting of the isolation of the transitory products, or an indirect approach, attempting the accumulation of oxalic acid from possible intermediates on the assumption that failure to produce the acid justified the conclusion that the substance did not represent a stage in the pathway to oxalic acid. The method employed for the solution of both aspects was to observe the formation or nonoccurrence of oxalic acid when the organisms were grown on media containing salts of the acids to be investigated as sole carbon source. The following groups of acids were utilized:

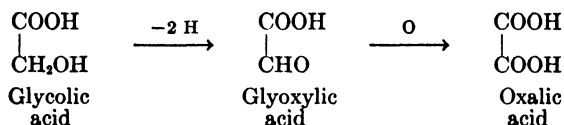
- (a) two-carbon acids: acetic and glycolic,
- (b) four-carbon acids: succinic, fumaric, and malic.

The wood-destroying molds employed in this investigation were numbers 1, 2, 3, 4 (see page 273). With the exception of 3, the organisms were able to produce oxalic acid, obtained as a salt, from all the acids.

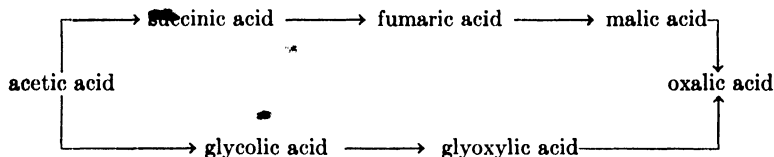
The earlier inability to identify or isolate oxalic acid as a metabolic product when the organisms were allowed to grow on a glucose medium prompted the consideration that, perhaps, the acid was formed, and, being in a free state, may have been further utilized by the organisms. It was found that oxalic acid could be used by organism 1 when in a free state, but not when added or present in the form of a salt. Perhaps, then, oxalic acid was formed when the organisms were grown on a glucose medium but, not being bound, it was further utilized by the molds.

The results attest to the production of oxalic acid from acetic acid via both pathways indicated. Although glyoxylic acid was not employed as a substrate since it can give rise *in vitro*, by the application of heat, to oxalic acid (16), the formation of oxalic acid from glycolic acid, together with the discoloration of resazurin in the glycolate

medium, presumed the intermediate formation of glyoxylic acid as follows:



The mechanism of acetate-oxalate transformation proceeds, consequently, along the following two pathways (55):



A relationship has been established between one group of metabolites, namely, ethyl alcohol, acetic acid, and acetaldehyde and oxalic acid. Since the same metabolic products have been obtained from cellulose and glucose by the action of the wood-destroying molds listed, we venture to present a mechanism for the breakdown of the cellulosic fraction of wood by the action of these molds. On the basis of our findings, therefore, we postulate the following picture of the fate of cellulose when attacked by these organisms:



An extension of the picture presented here was obtained by studying the metabolism of these molds when lactic, pyruvic, dimethylsuccinic, and dimethylfumaric acids were used as sources of carbon.

C. DISSIMILATION OF CELLULOSE BY WOOD-DESTROYING MOLDS

The action of the above-mentioned organisms on cellulose, in the form of filter paper and surgical cotton, was also studied. The formation of traces of reducing material was observed; the nature of this product, however, was not ascertained because of the minute quantities. The products obtained by the action of these molds on glucose are also metabolites of their action on cellulose, and in some cases on wood. Thus organisms 1, 2, 3, and 4 give rise to ethyl alcohol and acetic acid by their action on cellulose and glucose. Organisms 5, 6, and 9 give rise to oxalic acid by their action on wood, cellu-

lose, and glucose. Since the same metabolic products were obtained from wood, cellulose, and glucose, a mechanism postulated for the cellulosic fraction of wood deduced from a consideration of the phase sequence of glucose dissimilation would be justified.

Once again it appeared that a preliminary hydrolysis was required for the formation of the metabolites. In this connection it was thought of interest to examine the residual cellulosic material to determine whether there was an increase or a decrease in reducing power. If one were to assume a hydrolytic action similar to that of acids, there would be an increase in reducing power due to the formation of new reducing groups as a result of a splitting of 1,4 linkages. On the other hand, a cleavage of hemiacetal bonds might take place, with the result that the residual material would show a lower reducing power. Accordingly, surgical cotton was exposed to the enzymic attack by *Coniophora cerebella* over a period of sixty days and examined at intervals with the aid of the method of Hiller and Pacsu. Contrary to their results obtained with dilute acids it was found (58) that the reducing power of the attacked cellulose sample *increased* as the oxalic acid content increased. This would indicate that the mold effects a splitting of 1,4-glycosidic bonds giving rise to new reducing groups. Consequently, such action overshadows that which would effect a splitting of hemiacetal linkages.

V. Role of Lignin in Enzymic Degradation of Cellulose

In any consideration of the enzymic decomposition of cellulose, especially as a constituent of wood, it is important to bear in mind the possible influence of another constituent, lignin. It is generally assumed that in the development of the cell walls of woody tissues of vascular plants a change takes place whereby the cellulose becomes lignified. This consists in the addition to the cellulose of a substance or a group of compounds generally designated as lignin. Lignin is defined as that part of the wood which is obtained as an insoluble material when the carbohydrate-containing substances, remaining after removal of the tannins, resins, fats, etc. by extraction, are treated with strong mineral acids.

A. INFLUENCE OF LIGNIN ON DESTRUCTION OF CELLULOSE

It is common knowledge that plant materials are less readily decomposed as the extent of lignification increases. The attack of in-

tact wood and timbers by many basidiomycetes, however, is not greatly hampered by the presence of lignin.

At present a disagreement exists as to whether or not the presence of lignin has any retarding effect on the degradation of cellulose, as in wood. Thus, some bacteria which readily attack cellulose seem to be without effect on wood. Olson, Peterson, and Sherrard (61), on the one hand, have demonstrated that small amounts of lignin inhibit the attack on the cellulosic constituent. Only after practically all the lignin has been removed (the lignin content of wood must be less than 1%) was the cellulose attacked. On the other hand, Virtanen *et al.* (81,82) presented findings contrary to these. It was demonstrated that mechanical grinding is an important factor allowing for the decomposition of the cellulosic fraction of wood. The smaller the particle size in wood flour the greater was the extent of degradation of cellulose. The inability of certain bacteria to attack the cellulosic constituent of lignified materials is due, then, merely to the unsatisfactory penetration of the host. It was shown by these workers that lignin-rich pulps prepared in the laboratory are fermented by thermophilic bacteria. Spruce wood which had been only partly cooked with calcium bisulfite liquor and had retained most of its lignin could be decomposed. Partially delignified wood containing 24.4% lignin (compared with 28.3% originally present) could be destroyed to an extent of 35%, while wood still retaining 18.5% lignin could be decomposed to an extent of 80%. It would appear, then, that although a cooking procedure facilitated the subsequent attack, it is not absolutely necessary to remove the lignin.

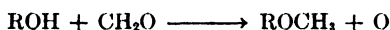
B. ORIGIN OF LIGNIN

While the question of the origin of lignin is still unsettled, there is some consideration of the possibility of its arising from carbohydrates. Many suggestions, some of which are purely speculative or are based on evidence of indirect or fragmentary character, have been advanced to explain the nature of the precursors of lignin and of the mechanism of the synthesis of lignin in the plant. Several investigators have advanced the view that lignin is formed by the plant from cellulose through a process involving methylation. It was thought that the plant could form lignin from pentoses. Klason (42) considered that dihydroxycinnamic acid, a compound which ultimately was converted

into lignin, was derived from two moles of pentoses in the following manner:



He considered the methylation of hydroxyl groups to take place through an interaction with formaldehyde. The formaldehyde, supposed to be formed as a result of a photochemical synthesis, methylated the hydroxyl groups as follows:



The hypothesis that lignin is formed by the plant from pentoses or pentosans was advanced by Rassow and Zschenderlein (75). They based this on the fact that hemp and flax rich in lignin were low in pentosans, and vice versa. Another school of investigators has suggested the possibility that soluble carbohydrates, pentoses, methyl pentoses, and hexoses may be used by the plant in the formation of lignin. Wislicenus (91) held the view that fructose was used in the synthesis of lignin. Phillips *et al.* (65) advanced the hypothesis that the plant builds up lignin directly from sucrose. In their studies on the lignification of barley and oat plants they showed that, although the percentage and absolute quantities of the lignin increased as the plant developed and matured, the absolute quantities of cellulose and pentosans did not decrease but rather increased. At no time was there any indication that the plant synthesized lignin at the expense of either of the above-mentioned carbohydrates. Although it is postulated that the plant builds up lignin directly from sucrose, no mention is made as to whether both the glucose and fructose residues of the disaccharide are used up for this synthesis, or fructose alone, as Wislicenus opined.

Essential in any synthesis of lignin is the production of a substance or substances possessing firmly bound methoxyl groups. These are assumed by Phillips *et al.* (66) to be formed in the splitting up of carbohydrates by a process of hydrolysis, oxidation, reduction, and dehydration.

On the basis of investigation carried out in our laboratories we are in a position to support the hypothesis of the formation of lignin from the carbohydrates of wood. Experimental evidence for our stand will be presented in the latter part of the discussion.

C. NATURE OF LIGNIN

Constituent Groups. While the chemical structure of lignin has not been categorically established, investigators in the field are agreed that the basal carbon skeleton of lignin is that of phenylpropane.

Although the composition of the lignins isolated by different means varies slightly, certain common data can be obtained from their study which would help to give an insight to the structure of this material. The evidence of the appearance of aromatic groups in lignin definitely establishes it as aromatic in character. Of the typical groups contained in lignin, the presence of aromatically bound methoxyl groups has been ascertained. It has been established that both phenolic and hydroxyl groups are contained in the

TABLE IV
ELEMENTARY COMPOSITION OF VARIOUS LIGNIN PREPARATIONS

Source	Method of isolation*	Carbon, %	Hydrogen, %	Investigators
Spruce	A	63.9	5.3	Klason
Fir	A	64.9	4.9	König
Beech	A	65.0	5.0	König
Spruce	B	62.4	6.4	Heuser, Schmitt, and Gunkel
Spruce	B	64.0	5.3	Hägglund
Fir	B	62.6	5.2	König and Rump
Beech	B	61.0	6.2	König and Rump
Spruce	C	63.9	6.0	Urban
Wood	C	64.7	5.5	Freudenberg and Harder
Flax	D	63.9	5.8	Powell and Whittaker
Larch	D	63.8	5.2	" " "
Pine	D	63.4	5.6	" " "
Spruce	D	64.0	5.5	" " "
Ash	D	63.2	5.6	" " "
Birch	D	63.2	5.5	" " "
Poplar	D	63.3	5.8	" " "
Corn cobs	D	62.5	5.0	Phillips
Oat hulls	D	64.4	5.2	"
Oat straw	D	61.8	5.4	Phillips and Goss
Wheat straw	D	61.8	5.5	" " "
Rye straw	E	63.0	5.6	Beckmann <i>et al.</i>
Corn cobs	E	61.1	5.8	Phillips
Oat straw	E	62.6	6.2	Phillips and Goss
Wheat straw	E	62.2	5.9	" " "
Spruce	F	63.6	5.7	Freudenberg <i>et al.</i>
Spruce	G	63.6	6.2	Brauns

* A, sulfuric acid method. B, fuming hydrochloric acid method. C, method of Urban. D, aqueous sodium hydroxide method. E, alcoholic sodium hydroxide method. F, Freudenberg cuprammonium method. G, alcohol extraction method without use of an acid.

molecule, and a variable proportion of them are methylated. One of the four hydroxyl groups considered to be available is phenolic in character, since it has been found that lignin preparations are soluble in dilute alkali from which they are precipitated by carbon dioxide (11,13,24). Although the presence of a carbonyl group has been the subject of dispute for many years, several investigators contributed evidence to support this view (11,17,71). Carboxyl groups are not regarded as being constituents of lignin. At present the molecular weight is considered to be about 840. A final decision as to the molecular structure of lignin awaits additional investigation.

Elementary Composition. The elementary composition of the lignin complex varies somewhat with the source and with the method employed for its isolation. Table IV presents a few of the results of the elementary analysis of various isolated lignins (64a).

D. NATURE OF LINKAGE BETWEEN CELLULOSE AND LIGNIN IN WOOD

1. Evidence of Physical Union

The probability of an inhibiting effect of the presence of lignin on the decomposition of the cellulosic constituent of plant materials, by the action of microorganisms, prompts the consideration of a union between them. Thus, a widespread controversial problem existing today is whether lignin in wood is in an intimate physical union with cellulose or is chemically bound with the cellulose. There are numerous investigators who support both sides of the question. Most of the evidence advanced to support either side, however, is fragmentary and indirect.

As early as 1920 Herzog and Jancke (29,30) showed that cellulose had a definite crystalline structure which was qualitatively the same regardless of its source. They reported for cellulose from cotton, ramie, wood, jute, flax, and linen identical powder patterns. They showed that lignified wood also gave a cellulose pattern. It would appear, then, that the lignin is adsorbed on the surface of the cellulose crystallite, probably, and not chemically bound.

In the same vein of thought, the unit cell of cellulose has been shown to be so small that the introduction of even a single phenylpropane nucleus would probably crowd it to the point of disintegration. The results of numerous x-ray studies have established that the cellulose

macromolecules in wood are crystallized against each other to form submicroscopic crystallites in which the dimensions of the unit cell are 10.2, 7.9, and 8.45 Å.

2. Evidence of Chemical Linkage

Evidence of a chemical nature which shed some light on the nature of the combination between cellulose and lignin was presented by Brauns (12). His investigations indicated that a small portion of the total lignin of wood (spruce) existed in a free state. Ethanol, used at room temperature, extracted up to 2-3% of the weight of wood used (or 8-10% of the total lignin content). Inasmuch as the conditions of the extraction were chemically inert and, too, the chemical studies established the extracted material as having the typical chemical properties of lignin, it was considered that this small fraction of the total lignin existed in a free state in the wood. Brauns gave it the name "native" lignin.

Additional extraction studies supported the contention that lignin originally does not exist chemically bound in wood. Thus, lignin can also be extracted by aqueous solutions of butanol and similar alcohols, provided the temperature is 120° or more (1). Bailey (2) digested aspen and jack pine woods in butanol-water mixtures buffered to prevent hydrolysis. By this method he was able to remove all of the lignin from aspen and 80% of the lignin from jack pine. These studies, then, support the view that the lignin extracted was not chemically bound with the cellulose of woods. The previously mentioned investigations of Virtanen *et al.* (81,82) also support the concept of a nonchemical union between lignin and cellulose. This group demonstrated the inability of certain thermophilic bacteria to attack the cellulosic fraction of wood, denying the existence of a chemical linkage.

While the research referred to above supports the assertion of the nonexistence of a chemical union between cellulose and lignin of wood, it has introduced a note of scepticism, and has even added some weight to the alternate hypothesis of a chemical linkage. Thus, the inability of Brauns to extract the remaining 90% of the lignin would indicate it is not in a free state. Too, Bailey was able to remove the residual 20% jack pine lignin, not extractable with the butanol-water mixture, by adding 2% caustic soda to the butanol-water mixture. This fraction of the jack pine lignin was considered

to be chemically bound. The fact that more drastic chemical procedures are required for the isolation of the total lignin would point to a probable chemical linkage with the cellulose.

Evidence for such a linkage between lignin and cellulose has been afforded by the research of several workers. Phillips (64) fractionally extracted the former from corn cobs by 2% alcoholic sodium hydroxide solution at room temperature, by 2% aqueous sodium hydroxide at 100° and 135°, and finally by 4% aqueous sodium hydroxide at 180°. Each method of extraction was continued until no further lignin was obtained, before the next method in the series was employed. On the basis of these studies Phillips concluded that the lignin in corn cobs is unequally combined with the carbohydrates, part of it being loosely held, probably in the form of an ester, and the remainder being more firmly held, possibly in the form of an ether-like union. It would appear that there is no alkali lignin present in corn cobs in the free state, for extraction with a solvent such as a 2:1 acetone-ethanol mixture, which is an excellent solvent for free alkali lignin, fails to yield any lignin.

The investigations of Harris, Sherrard, and Mitchell (25) added weight to the concept of the existence of a chemical linkage. On methylating maple wood with dimethyl sulfate and caustic soda, they found that the lignin isolated from the methylated wood was unchanged in methoxyl content, although the concentration of alkali was sufficient to allow for the methylation of the isolated lignin. However, upon first subjecting the wood to a mild hydrolysis with 1% acid, the lignin was methylated *in situ*. This could mean that the lignin hydroxyl groups were masked by the union of the lignin with other constituents of the maple wood, and these were liberated by the hydrolysis.

Weak evidence to support a lignin-cellulose chemical linkage was offered by Ploetz (68). The digestive juices of the Weinberg snail (*Helix pomatia*) were known to contain the enzymes cellulase, lichenase, and cellobiase, and the purified solutions were capable of converting cellulose to a water-soluble condition. Regenerated cellulose was also found to be solubilized with ease. On the other hand, the carbohydrates in mature wood were found to be resistant to the action of the enzymes. Ploetz inferred that this resistance was caused by mechanical factors or by the protection afforded by a combination with lignin. Later research seemed to support the latter reason.

Finely divided lindenwood was degraded with cupri-ethylenediamine solution, and the extract acidified. The precipitate from the acidified extract was very easily degraded by enzyme preparations, giving residues whose composition corresponded to a lignin:carbohydrate ratio of 1:1. The residual wood which was insoluble in the solution was attacked only with difficulty, the degradation ceasing when the ratio of lignin to carbohydrates reached 1:3. Ploetz deduced that the lignin of wood consists wholly, or at least in part, of "lignin-carbohydrate" combinations, the 1:3 combination being a higher structural unit from which the 1:1 complex is derived. In evaluating the results of this investigation it must be borne in mind that the lignin may have been structurally altered by the cupri-ethylenediamine treatment.

Moreover, while working with cottonseed hulls, Smith and Purves (79) obtained additional indirect evidence to support the nonexistence of a lignin-cellulose chemical linkage. Crushed cottonseed hulls, extracted with alcohol-benzene, were ground in a ball mill and the less fibrous portion was separated by sieves and by sedimentation from water into coarse and fine fractions. These were analyzed by standard methods for pentosan, uronic anhydride, and lignin. While the original hulls contained pentosan, uronic anhydride, and "lignin" to the extent of 27, 6.5, and 25%, the corresponding figures were approximately 3, 9, and 50% for the finest fraction and 40, 6, and 24% for the coarser ones. The "lignin" spoken of was insoluble in 72% sulfuric acid and differs from similarly prepared wood lignin in that it is methoxy free. The results obtained indicated that the pentosans and "lignin" were present as independent separate chemical entities, both of which contain uronic anhydride.

While admitting that some evidence has been presented which corroborates both the existence of chemical linkage and a physical lignin-carbohydrate union, the balance would not seem to support the presence of a chemical linkage. One can take the evidence of Brauns (11) to mean that at least part of the lignin of wood is in a free state. At least, then, we cannot deny that there is a basis for the nonchemical union concept. The evidence presented to support the chemical linkage theory is indirect in nature. And, only because it does not favor support of the hypothesis of a nonchemical union is it used to uphold the alternate view.

Thus, Phillips (64) assumes that the lignin in corn cobs is unequally

combined, possibly in the form of an ester, and the rest more firmly held, probably in the form of an ether-like combination. This conclusion is based on the fractional extraction from corn cobs of lignin by the use of varying concentrations of caustic soda at several temperatures. It is difficult to reconcile his conclusions with findings of other investigators, however, since it has been established that lignin does not contain any carboxyl groups which could allow for ester formation.

Among those investigators who have accepted the view that lignin is chemically linked with cellulose or with the other carbohydrate materials, much difference of opinion prevails regarding the manner in which this union exists. One school of thought, of which Erdmann (18) and Lange (45) were members, assumed that an ester-like linkage exists. On the other hand Hoppe-Seyler (36), Grafe (23), and Mehta (48) held the view that there exists an ether-like union.

Taking into consideration the reactive groups in cellulose and lignin, the possibility narrows down to one type of linkage, that of an ether-like type. There are strong reasons against the alternate ester-type linkage. A linkage of the ester type is only possible when one of the constituents possesses marked acid properties. This is not the case with lignin. Carboxyl groups have been shown not to exist in the lignin molecule.

The ether-type linkage which could result from the hydroxyl groups contained by both classes of compounds would probably be too stable to be cleaved by the not-so-drastic procedures by which lignin is isolated. Assuming an acetal type linkage between lignin keto and carbohydrate hydroxyl groups, such a union would be improbable, too, because bonds of this type are usually stable to alkali. Much lignin is extracted from plant materials by the use of alkali in mild conditions. And for the same reason glycosides engaging aliphatic hydroxyl groups in the lignin molecule are unlikely, although Mehta (48) favors the view that lignin is present in combination with cellulose and certain polysaccharides as a glucoside.

The views of both schools of thought concerning the linkage between lignin and cellulose in wood have been presented. It would appear that the balance is in favor of the nonexistence of a chemical linkage. For, while it cannot be denied that at least part of the lignin is in the free state, the inability to isolate the remaining part of the

lignin by the same chemically inert method may be due to a physical phenomenon.

Unless one assumes the presence of an enzyme system capable of splitting the hypothetical cellulose-lignin chemical linkage, the action of wood-destroying molds on wood, free cellulose, and glucose producing identical breakdown products would support the contention of the nonexistence of a chemical union.

An interesting and plausible interpretation concerning the morphological relationship of lignin and cellulose in the cell wall of fir trees is offered by Kisser (40). He assumes the lignin to fill in the voids in an interlocking capillary system which exists in the framework of the wood. Thus, the inability of *Fomes hartigii* to attack rapidly the tangential walls of fir is due to this low degree of lignification.

E. CONSTITUTION OF LIGNIN

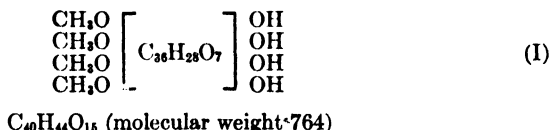
Although frequent mention of lignin has been made in the preceding part of this presentation, it should be kept in mind that knowledge of the chemistry of this substance is incomplete and any attempt to assign a definite constitutional formula to lignin is unwarranted. At the present time no clear-cut picture could demonstrate its molecular structure. A number of structural formulas have been proposed but as yet none has been accepted as truly representative of the compound.

In any attempt to visualize lignin structurally it is equally agreed that the compound should have a molecular weight of about 840, several hydroxyl groups, several methoxyl groups, one enolic, one phenolic, and one carbonyl group. Too, it is generally accepted that lignin may in large part be built up of phenylpropane building units with phenolic hydroxyls or methoxyls in the 3,4 positions.

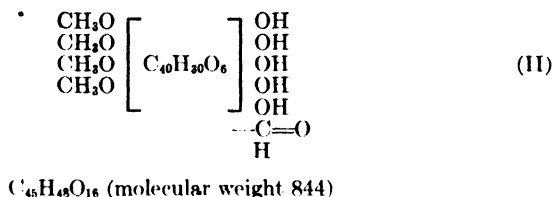
It is agreed that of the *chemical* methods employed to obtain lignin no method is known by which it can be isolated unchanged. By all chemical methods applied, a lignin preparation is obtained which is no longer identical with native lignin. Brauns' native lignin would appear to be the exception.

Several lignin samples prepared in diverse ways have been obtained which approximate these demands and which are in themselves similar. Paschke (63) isolated lignin from straw by digestion with sodium carbonate. The empirical formula $C_{40}H_{46}O_{15}$ was assigned to this product. In 1921 Beckmann and Liesche (and Lehmann) (5) extracted lignin from winter rye straw using alcoholic sodium hydroxide.

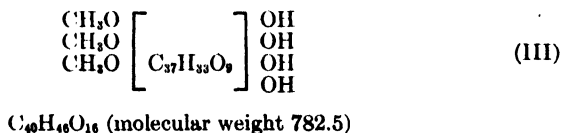
Its molecular weight was of the order of 800 and its composition corresponded to the formula $C_{40}H_{44}O_{15}$, this unit containing four hydroxyl and four methoxyl groups. Its detailed formula may be written as in I.



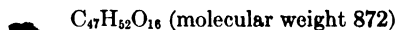
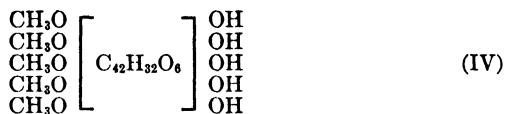
Powell and Whittaker (71) isolated lignin from flax shives by digestion with sodium hydroxide. The formula for flax lignin which corresponds to all the analytical data is $C_{45}H_{48}O_{16}$. The molecule contains four methoxyl groups and five hydroxyl groups capable of acetylation. That three of these groups are phenolic in character is shown by the fact that not more than three additional methoxyl groups can be introduced. The compound reduced Fehling's solution on warming, thus indicating the presence of an aldehydic group in the molecule. Therefore, the formula for this lignin is II.



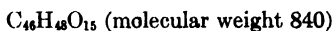
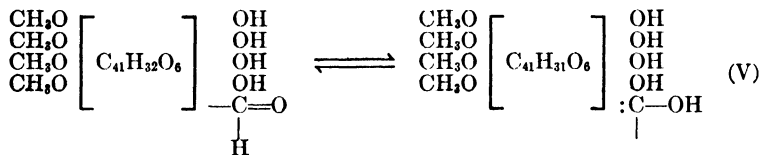
Phillips (64) isolated lignin from corn cobs employing the same medium. The data obtained indicate that his lignin is a fairly homogeneous substance or mixture of closely related isomers. Analysis of this material gave the following results: C, 61.08%, 61.24%; H, 5.91%, 5.85%; molecular weight, 755. From a consideration of all the data, the formula $C_{40}H_{46}O_{16}$ was assigned as the most probable one for this lignin fraction from corn cobs. The molecular weight and the percentage composition calculated for a compound of this formula are: molecular weight: 782.5; C, 61.36%; H, 5.92%. Therefore, the extended formula is III.



A similar lignin preparation was obtained by Brauns and Hibbert (13), by extracting spruce wood meal with absolute methanol in the presence of hydrochloric acid. From the elementary analysis they arrived at the empirical formula $C_{47}H_{52}O_{16}$. In a deployed form it was represented by IV.



The above-designated lignin preparations all show some similarity to Brauns' native lignin, although it is assumed that they underwent some change during the various procedures of isolation. Inasmuch as the conditions of the extraction were chemically inert, it is assumed that this small fraction of the total amount of the lignin of wood is truly representative of the unchanged native lignin. Analytical data for native lignin point to an empirical formula $C_{48}H_{46}O_{15}$ and a molecular weight of 840. The presence of four methoxyl groups and five hydroxyl groups was indicated. One of the hydroxyl groups was assumed to be enolic in character. Thus, Brauns claimed to have established the presence of a carbonyl group by preparing a hydrazone (13.3% methoxyl). His formulation for this native lignin involves a keto-enol isomerism which is represented by V. Such a formulation agrees well with the analytical data enmassed.



F. CONTRIBUTIONS OF ENZYME STUDIES TO THE LIGNIN PROBLEM

1. Biochemistry of *Merulius lacrymans*

Prior to Brauns' (1939) successful attempt to isolate lignin from wood in an unaltered state by the use of a chemically inert extraction process, Barton-Wright and Boswell (3) obtained a lignin preparation very similar in constitution to native lignin. These workers were

concerned with the biochemistry of dry rot in wood. Primarily they were interested in an investigation of the products of decay of spruce wood rotted by *Merulius lacrymans*, a brown-rot type of wood-destroying fungus. Being a brown-rot organism, it was expected that the cellulosic fraction of the wood would be consumed. The results of Table V substantiate this.

TABLE V

Treatment	Sound wood,* %	Decayed wood, %
Extracted by benzene	1.70	1.48
alcohol	1.60	19.80
water	2.50	8.00
5% NaOH in cold	3.80	19.10
Cellulose	55.00	9.88
Lignin	28.00	40.90
Mannan	7.60	0.00
Galactan	0.10	0.00
<i>Total</i>	100.30	99.16
Ash	0.86	0.61
Natural moisture	10.00	17.30

* Calculated on the basis of ash-free material dried at 100°.

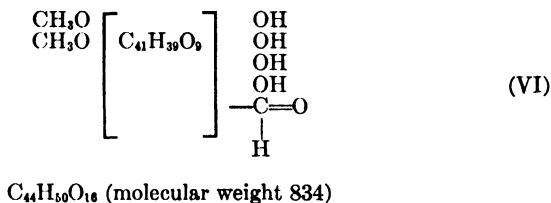
The removal from wood of cellulose (and of the noncellulosic carbohydrates) by the action of this fungus on Norwegian spruce wood suggested a method by which lignin in its original free state could be isolated from wood. It could be presumed that such a preparation might be identical with Brauns' lignin. A lignin body obtained from wood which had been decayed by the wood-destroying fungus *Merulius lacrymans* to the extent indicated in the above table was obtained. It seems to us that a definite similarity in structure between the two samples of lignin obtained by Brauns and the British authors exists.

M. lacrymans was able to destroy the major part of the wood, leaving the lignin component unaffected. A homogeneous lignin preparation was extracted from the rotted wood with alcohol. After removal of the solvent, a product was obtained which was soluble in acetone, glacial acetic acid, and sodium hydroxide solution, and insoluble in benzene and low-boiling petroleum ether. It was precipitated from glacial acetic acid by the addition of water or from sodium hydroxide solution by the addition of hydrochloric acid. The substance reduced Fehling's solution and the presence of an aldehyde group was

Author	Source	Method of isolation	Molecular weight	Formula
Paschke (63)	Straw <i>sega.</i>	Digestion with Na ₂ CO ₃	765	C ₄₀ H ₄₈ O ₁₅
Beckmann and Liesche (5)	Winter rye straw	Alc. NaOH	764	$\begin{array}{c} \text{CH}_3\text{O} \\ \text{CH}_3\text{O} \\ \text{CH}_3\text{O} \\ \text{CH}_3\text{O} \end{array} \left[\begin{array}{c} \\ \\ \\ \end{array} \right] \begin{array}{l} \text{OH} \\ \text{OH} \\ \text{OH} \\ \text{OH} \end{array}$ <p style="text-align: center;">C₃₈H₂₈O₉ C₄₀H₄₄O₁₅</p>
Powell and Whittaker (71)	Flax shives	Aq. NaOH	844	$\begin{array}{c} \text{CH}_3\text{O} \\ \text{CH}_3\text{O} \\ \text{CH}_3\text{O} \\ \text{CH}_3\text{O} \end{array} \left[\begin{array}{c} \\ \\ \\ \end{array} \right] \begin{array}{l} \text{OH} \\ \text{OH} \\ \text{OH} \\ \text{OH} \\ \text{OH} \\ -\text{C=O} \\ \text{H} \end{array}$ <p style="text-align: center;">C₄₀H₃₀O₈ C₄₈H₄₈O₁₆</p>
Phillips (64)	Corn cobs	Aq. NaOH	782.5	$\begin{array}{c} \text{CH}_3\text{O} \\ \text{CH}_3\text{O} \\ \text{CH}_3\text{O} \end{array} \left[\begin{array}{c} \\ \\ \end{array} \right] \begin{array}{l} \text{OH} \\ \text{OH} \\ \text{OH} \end{array}$ <p style="text-align: center;">C₃₇H₃₃O₉ C₄₀H₄₆O₁₆</p>
Brauns and Hibbert (13)	Spruce wood	CH ₃ OH with HCl as catalyst	872	$\begin{array}{c} \text{CH}_3\text{O} \\ \text{CH}_3\text{O} \\ \text{CH}_3\text{O} \\ \text{CH}_3\text{O} \\ \text{CH}_3\text{O} \end{array} \left[\begin{array}{c} \\ \\ \\ \end{array} \right] \begin{array}{l} \text{OH} \\ \text{OH} \\ \text{OH} \\ \text{OH} \\ \text{OH} \end{array}$ <p style="text-align: center;">C₄₂H₃₂O₈ C₄₇H₅₂O₁₆</p>
Brauns (11)	Spruce wood	Extraction with alcohol	840	$\begin{array}{c} \text{CH}_3\text{O} \\ \text{CH}_3\text{O} \\ \text{CH}_3\text{O} \\ \text{CH}_3\text{O} \end{array} \left[\begin{array}{c} \\ \\ \end{array} \right] \begin{array}{l} \text{OH} \\ \text{OH} \\ \text{OH} \\ \text{OH} \\ -\text{C=O} \\ \end{array}$ <p style="text-align: center;">C₄₁H₃₂O₈ C₄₆H₄₈O₁₅</p>
Barton-Wright and Boswell (3)	Spruce wood	Alcohol extract of decayed wood	834	$\begin{array}{c} \text{CH}_3\text{O} \\ \text{CH}_3\text{O} \end{array} \left[\begin{array}{c} \\ \end{array} \right] \begin{array}{l} \text{OH} \\ \text{OH} \\ \text{OH} \\ \text{OH} \\ -\text{C=O} \\ \text{H} \end{array}$ <p style="text-align: center;">C₄₁H₃₀O₉ C₄₄H₅₀O₁₆</p>

composition $C_{44}H_{80}O_{16}$. Such a compound requires: C, 63.3%; H, 5.99%. An analysis for methoxyl content showed the product to contain two methoxyl groups (CH_3O , 9.21%). The presence of four hydroxyl groups was indicated by the preparation of a tetrabenzoyl derivative (found: C, 69.0%; H, 5.6%). $C_{44}H_{46}O_{12}(OCOC_6H_5)_4$ requires C, 69.1%; H, 5.28%. The product appeared to be perfectly homogeneous since all attempts to separate it into different fractions failed.

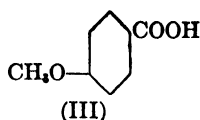
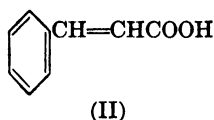
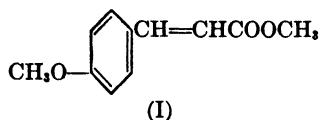
Uninfected wood treated in a similar way did not give rise to an alcohol-soluble fraction. It must be assumed, therefore, that the above product is the "result" of the action of *Merulius lacrymans* on spruce wood. The extended formula of the compound can be written as in VI.



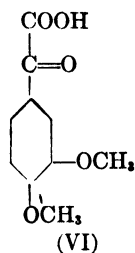
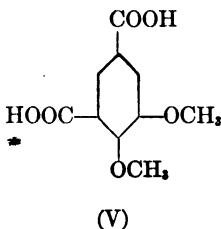
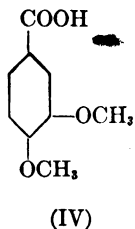
In Table VI data are presented for various "similar" lignin preparations. Although the isolation methods vary, the differences among the samples do not appear to be too great. It would be conceivable, then, that the efforts of additional research on any one of these preparations should contribute definitely toward the elucidation of the complete chemical structure of lignin.

2. Biochemistry of *Lentinus lepideus*

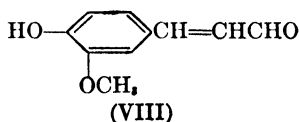
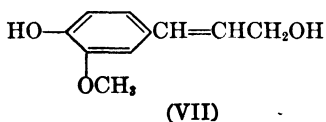
In that part of the discussion dealing with the possible origin of lignin some speculation was presented as to the possibility of its arising from the carbohydrates of wood. During the course of the study of the biochemistry of wood-destroying molds an interesting observation was made which would support such a hypothesis. Reference is made in the literature (7) to the formation of several aromatic compounds containing methoxy groups by the action of a wood-destroying fungus, *Lentinus lepideus*, on white Scots pine wood. The products formed are methyl *p*-methoxycinnamate (I) and the methyl esters of cinnamic acid (II) and of anisic acid (III).



These compounds are similar in structure to several breakdown entities of lignin. Thus, a few fission products of the aromatic class which have been isolated from lignin are veratric acid (IV), isohemipinic acid (V), and veratroyl formic acid (VI).



As early as 1897, Klason (41) was of the opinion that lignin is a condensation or polymerization product of coniferyl alcohol (VII). Later (43) he suggested that coniferyl aldehyde (VIII) may be the



basic building stone of lignin. The resemblance of the compounds is apparent. Since methyl *p*-methoxycinnamate and the other products are similar in structure to the lignin fission units, one could assume that they arose from the lignin portion of the wood.

It has been demonstrated (56) that *Lentinus lepideus* gives rise to methyl *p*-methoxycinnamate when grown on media containing glucose or xylose. Assuming a preliminary hydrolytic action by the fungus, it would appear that the ester arises from the cellulosic and/or pentosan fractions of the wood rather than from the lignin, and, consequently, its appearance from wood corroborates the theory also of the degradation of wood via an introductory hydrolysis when it is decomposed by microorganisms. Furthermore, the significant finding that the action of the fungus on xylose gives rise to the same product prompts, of course, a consideration as to a mechanism of its formation. Since the organism is a perfect alcoholic fermenter, it would

seem that there is the transient formation of a two-carbon compound which acts as a switchboard for the building up of the ester. The ease with which ethyl alcohol, added to or formed in the media, or glycerol was dehydrogenated when serving as carbon sources suggested acetaldehyde (53) as the "labile" transient compound. This was found to be a fact (57) namely, when the fungus was grown on an ethyl alcohol-containing medium it gave rise to the ester. Moreover, when Dimedon was incorporated in such a medium in a parallel experiment, the acetaldehyde-Dimedon compound was obtained; the formation of this compound prevented the utilization of the acetaldehyde in the synthesis of the ester. The fact, then, of the formation of methyl *p*-methoxycinnamate from wood, glucose, or xylose supports the hypothesis that the carbohydrate fraction of wood serves as the source of the lignin.

By means of enzyme studies the phase sequence of the degradation of cellulose in the course of wood decay caused by molds has been elucidated. Similar investigations have also helped shed light on other coordinated problems. Thus, the application of enzymes to the preparation of lignin presents a good outlook for obtaining *unaltered* lignin, the study of which might enhance the clarification of its constitution. Moreover, continued research in this direction should further assist in understanding the mechanism of lignification, since it has for the first time been experimentally demonstrated that an enzymically formed methylated aromatic compound might serve as a transition product between carbohydrate degradation and lignification.

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SYNTHESIS OF LIPIDES

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I. Introduction

Nearly ninety years have passed since the first convincing experimental evidence was obtained of the synthesis of fat from carbohy-

drate. Since that time numerous investigators have studied this problem; and although our knowledge in this field has now been greatly extended many details of the mechanism of the process are still lacking, partly because of the inherent difficulties of the subject. While the great progress in our knowledge of carbohydrate metabolism was due in the main to the discovery of cell-free extracts with which most, if not all, the individual reactions involved could be examined, the study of the synthesis of lipides from nonlipide substrate is still confined to whole cells or organisms.

In the following, a short account of our present knowledge of the synthesis of various lipides and their components, *i.e.*, fatty acids, glycerides, phospholipides, and some wax constituents, will be presented, and the mechanism of the syntheses will be discussed in the light of recent evidence. A complete list of references is not given; most can be found in such excellent reviews as the papers and monographs of Leathes and Raper (67), Smedley-Maclean (119), Longenecker (73), Bernhauer (13), McHenry (82), and Hilditch (50).

II. Synthesis of Fatty Acids

A. EVIDENCE OF SYNTHESIS OF FATTY ACIDS FROM CARBOHYDRATE

Although the synthesis of fat from carbohydrate has been known for a long time (fattening of animals on a high carbohydrate diet), Lawes and Gilbert (66) were the first to supply convincing evidence for the conversion of carbohydrate to neutral fat. Their experiments were based on a balance sheet of carbohydrate, protein, and fat ingested, and of fat, carbohydrate, and protein formed in pigs. Further evidence was supplied by a number of workers. Morgulis and Pratt (87) demonstrated fat synthesis in dogs, and also showed that fat formation is accompanied by a respiratory quotient higher than 1.0. In "well-nourished" swine, fat is synthesized at a rate of 1% of body weight daily (102,147). Benedict and Lee (11) studied in detail fat formation in geese. Schoenheimer and Rittenberg (116), using deuterium as labeling agent, demonstrated in an elegant way fat formation in rats, and showed that these animals can rapidly synthesize fatty acids when fed on a practically fat-free diet (see Fig. 1). In mice on a high-carbohydrate diet the rate of synthesis of fatty acids is about 1 g. per day (Stetten and Grail, 133).

In higher plants, the investigations of du Sablon (110) suggest fat

synthesis from carbohydrate. In ripening seeds the increase of fat is accompanied by a simultaneous decrease in carbohydrate content. Eyre (37) studied the formation of fat in the *Linum usitatissimum*, and found a rapid increase of the fat content of the seeds (see Fig. 2.).

Numerous microorganisms have been shown to be capable of synthesizing large amounts of fat from carbohydrate, e.g., yeasts, molds, bacteria (for a detailed list of references see the review of Bernhauer, 13). Some of these organisms are capable of accumulating 50% and more of their dry weight in fat. In some yeasts, fat formation from carbohydrate proceeds rapidly, as shown in Figure 3

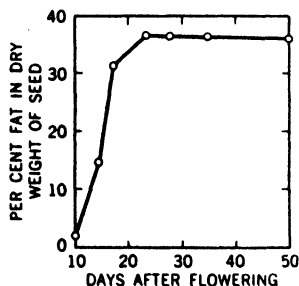


Fig. 2. Fat formation in ripening linseed (37).

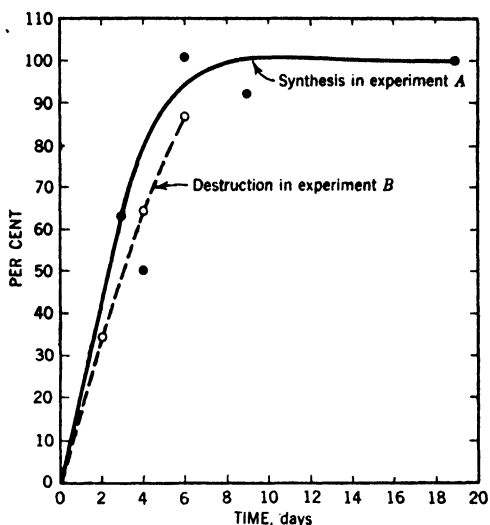


Fig. 1. Synthesis and destruction of fatty acids in mice (116).

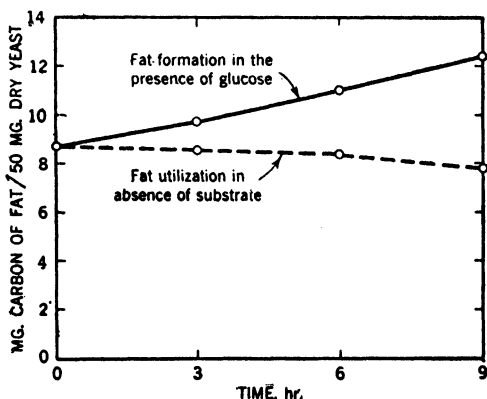
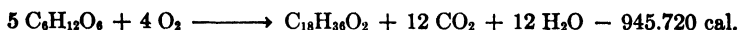


Fig. 3. Fat formation from glucose in *Torulopsis lipofera* (60).

for the yeast *Torulopsis lipofera*. This yeast forms fat at a rate of 4–11% of its dry weight in 5 hours, i.e., the synthesis of fat proceeds several times faster than in the above-mentioned pigs.

B. RESPIRATORY QUOTIENT AND EFFICIENCY OF CONVERSION OF CARBOHYDRATE TO FAT

The synthesis of fatty acids from carbohydrate is a reductive, endothermic process, as, for example:



(20). The formation of fatty acids must therefore necessarily be coupled with the oxidation of an equivalent amount of other substrate, most probably carbohydrate. From the above equation it also follows that the respiratory quotient, R.Q., *i.e.*, CO_2 formed/ O_2 used, during the process will be higher than 1.0. Numerous authors have demonstrated high respiratory quotients during fat formation. Thus Wesson (146) observed in rats fed on dextrin with a small amount of casein an R.Q. of 1.5 to 2.0. During the formation of fat in ripening castor oil seed and olives Gerber (43) found an R.Q. of 1.51, but only during the fat-forming stage. In yeast, in the presence of carbohydrate, the R.Q. often rises above 1.0, and in the fat yeast, *T. lipofera*, during fat formation an R.Q. of 1.15 to 1.50 was observed (60).

Further consideration of the above equation shows that the R.Q. could also be used as a measure of the efficiency of the synthesis of fatty acids from carbohydrate. Recently, two additional terms have been suggested to express the efficiency of this process, *i.e.*, by relating the utilization of the substrate to the amount of fat formed. Rippel (106) introduced the term *fat coefficient*, which expresses the percentage of fat formed from the weight of substrate used. Independently, Kleinzeller (60) used the term *conversion coefficient* to denote the percentage of carbon of substrate converted to carbon of fat. Table I shows the relationship of R.Q., conversion coefficient,

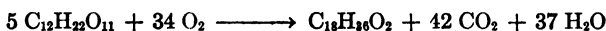
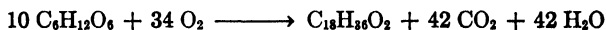
TABLE I

R.Q., CONVERSION AND FAT COEFFICIENTS CALCULATED FOR VARIOUS EQUATIONS OF CONVERSION OF HEXOSE TO STEARIC ACID

Reaction	R.Q.	Conv. coeff., %	Fat coeff., %
$5 \text{ C}_6\text{H}_{12}\text{O}_6 + 4 \text{ O}_2 \longrightarrow \text{C}_{18}\text{H}_{36}\text{O}_2 + 12 \text{ CO}_2 + 12 \text{ H}_2\text{O}$	3.0	60	31.7
$6 \text{ C}_6\text{H}_{12}\text{O}_6 + 10 \text{ O}_2 \longrightarrow \text{C}_{18}\text{H}_{36}\text{O}_2 + 18 \text{ CO}_2 + 18 \text{ H}_2\text{O}$	1.8	50	26.3
$8 \text{ C}_6\text{H}_{12}\text{O}_6 + 22 \text{ O}_2 \longrightarrow \text{C}_{18}\text{H}_{36}\text{O}_2 + 30 \text{ CO}_2 + 30 \text{ H}_2\text{O}$	1.36	37.5	19.7
$10 \text{ C}_6\text{H}_{12}\text{O}_6 + 34 \text{ O}_2 \longrightarrow \text{C}_{18}\text{H}_{36}\text{O}_2 + 42 \text{ CO}_2 + 42 \text{ H}_2\text{O}$	1.24	30.0	15.8

and fat coefficient for various equations of the synthesis of stearic acid from hexose.

In the following both the above terms will be used. These terms are directly related only when the fat formation from substrates of the same empirical formula is calculated. Thus, for the equations:



the respiratory quotients and the conversion coefficients remain constant, while the fat coefficients are 15.78 and 16.61, respectively. It is therefore suggested that the term fat coefficient be used when comparing the fat formation from the same substrate. This term has recently been widely used, especially when calculating the overall fat formation under conditions when the organism both grows and accumulates fat. Under these conditions the use of this term is invaluable. The conversion coefficient will be useful when comparing the formation of fat from different substrates, since it allows direct comparison of the results obtained.

Generally speaking, it is possible to state that the conversion coefficient found in living cells which form fat is always considerably lower than the highest conversion coefficient theoretically possible (on the assumption that in the course of fat synthesis carbohydrate is first broken down to two molecules of C_2 compounds and two molecules of carbon dioxide, and that the C_2 compounds are then condensed to fatty acids, the highest conversion coefficient would be 66.7%). This is due to the fact that simultaneously with fat formation other metabolic processes take place which derive their energy requirements from the substrate. This aspect will be discussed below in connection with carbon balance sheets during fat formation. The only experimental finding in which the conversion coefficient reached the theoretical limit is the observation of Terroine and Bonnet (139). These authors measured the caloric value of glucose used, and fat formed, in *Aspergillus niger*, and then arrived at a proportion of 4.38 molecules of glucose used per molecule of oleic acid formed; the conversion coefficient according to these experiments would be 71%. The majority of experimental results appear to confirm the view that the efficiency of the conversion of carbohydrate to fat does not approach the theoretical figure of 66.7. Thus, in fattening pigs, the fat coefficient usually found is about 24–25% (i.e., conversion coef-

ficient about 47%). In resting cells of *T. lipofera*, Kleinzeller (60) found conversion coefficients of 20–30%; Cada (21) found in *Endomyces vernalis*, during fat formation in absence of assimilable nitrogen, a fat coefficient of 23.4%, but with a slight simultaneous loss of cell protein. The same author found, using a strain of *Mucor*, fat coefficients between 10–12.

TABLE II
CARBON BALANCE IN *Torulopsis lipofera**

Analysis	Initial	After 5 hr.	Formed or used, mg.	C used, mg.	C formed, mg.
Glucose	3290 mg.	1540 mg.	–1750	700	—
Carbon dioxide	—	1105 mg.	+1105	—	301
C in medium (nonvolatile)	1315 mg.	634 mg.	–681	(681)	—
Ethanol	—	10 mg.	+10	—	5
Lactic acid	—	—	—	—	—
Ketonic acids	—	—	—	—	—
Yeast (dry wt.)	3146 mg.	3923 mg.	+774	—	—
C in yeast	1533 mg.	1903 mg.	+370	—	370
N in yeast	164 mg.	169 mg.	+5	—	—
Unsaponifiable	56 mg.	62 mg.	+6	—	(158)
Fatty acids	706 mg.	908 mg.	+202	—	—
I.V.	82.3	77.3	—	—	—
Mean mol. wt.	270	275	—	—	—
Carbohydrate in yeast	—	—	—	—	—
Insol. in H ₂ O	332 mg.	635 mg.	+302	—	(151)
Insol. in 60% EtOH	216 mg.	278 mg.	+62		
Insol. in 80% EtOH	40 mg.	16 mg.	–24		
<i>Total</i>				700	676 (i.e., 97%)

* Figures in parentheses indicate that they have already been included in the balance under a different heading.

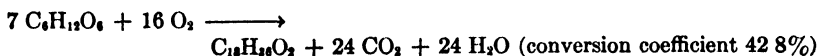
The fat or conversion coefficients reported here do not allow conclusions to be drawn as to the stoichiometric proportions of fatty acids synthesized from carbohydrate. As shown below, simultaneously with fat formation other metabolic processes take place in the resting cell, such as accumulation of carbohydrate. The fat or conversion coefficients found can therefore be regarded only as over-all expressions. Detailed carbon balance sheets may, however, help to arrive at true figures which might allow the relation of glucose converted to fatty acids to be formulated more clearly. The only carbon balance sheet during fat formation was done on *T. lipofera*, showing that 33–43% of the carbon of glucose was oxidized to carbon dioxide.

A considerable increase in the weight of the cells took place, due to an increase in both fatty acids and carbohydrate; little if any unsaponifiable material was formed under these conditions (see Table II). An accumulation of cell carbohydrate during fat formation was also demonstrated by Smedley-Maclean and associates (77,121).

Rippel (106), on the basis of theoretical considerations, arrives at the conclusion that in growing microorganisms the fat coefficient cannot exceed 15%. He takes into account not only the formation of fat, but also the formation of protein and other components of the cell. In experiments in which both growth and fat formation occur, the fat coefficient has until recently been found to be considerably lower than the theoretical maximum of Rippel. Thus Lindner (quoted by Fink *et al.*, 38) found in *Endomyces vernalis* a fat coefficient of 7.2. Myrbäck *et al.* (36,92) found in various yeasts, fat coefficients varying between 5–11%. More recently, however, considerably higher results were obtained. In the experiments of Starkey (129) yeast strains No. 72 and 74 had fat coefficients of 12–13%. Very recently, Enebo *et al.* (35) found fat coefficients as high as 18, with a fat content of the yeast *Rhodotorula gracilis* 60%, and explained that Rippel in his calculations did not expect such low protein content in the microorganism when the fat content reaches such high figures. Kleinzeller, Vihan, and Bass (61) obtained with *T. lipoferula*, and with yeasts No. 72 and 74 (courtesy of Dr. Starkey), fat coefficients between 11–15% on inorganic cultivation media, and, occasionally, also figures approaching those of Enebo *et al.* (*e.g.*, 17.5% with a fat content of 50% in the dry yeast). The Swedish authors calculate from their experimental data that, on an average, 4.55 g. glucose are consumed in the formation of 1 g. fat from carbohydrate, irrespective of whether fat yeasts (*e.g.*, *R. gracilis*) or a yeast with a low fat content (*T. utilis*) are considered. They base their calculation on an equation of Sperber (125):

$$\text{yeast yield (\%)} = \frac{100 \times \text{g. newly formed yeast}}{2.0(\text{g. protein} + \text{g. carbohydrate}) + y \times \text{g. fat}}$$

and assume that 2.0 g. glucose is consumed in the formation of 1 g. protein or carbohydrate. From the data of these authors, the real conversion coefficient would be 41.2%, approximately corresponding to the equation:



C. CONDITIONS OF FAT FORMATION

The conditions of fat formation have been mostly studied in microorganisms, since these lend themselves far better for such experiments than animals or higher plants. Many microorganisms readily form fat when carbohydrate is the only substrate used. So far, no experimental evidence has been supplied which would invalidate the assumption that the main pathway of the synthesis of fatty acids from carbohydrate is essentially the same in all living cells. The observations on microorganisms in that respect may therefore also be valid for these processes in animal or higher plant cells.

1. Oxygen

So far no convincing evidence has been supplied that fatty acids can be synthesized under anaerobic conditions. On the other hand, a number of authors have shown that fat synthesis from carbohydrate is enhanced by a good supply of oxygen. Nägeli and Loew (90) were the first to demonstrate the formation of fat in yeast, and also found that this proceeds only in the presence of oxygen. Smedley-Maclean in a number of papers (118,120,121) showed that oxygen is essential for fat synthesis in baker's yeast. *Torulopsis lipofera* does not metabolize carbohydrate at all under anaerobic conditions, but readily synthesizes fat in the presence of oxygen. The fat formation in this yeast is not further enhanced by increasing the concentration of oxygen over that in air. Some microorganisms, which have been studied in some detail with the view of biological fat synthesis for technical purposes, e.g., *Endomyces vernalis*, or *Fusarium lini* (27), also form considerable amounts of fat only when sufficient oxygen is supplied, either by cultivating the organisms on large surfaces, or providing them with oxygen under pressure.

2. Temperature

The optimal fat formation from carbohydrate in microorganisms generally coincides with their optimal growth temperature. The chemical characteristics of the fat formed may, however, vary with temperature. This aspect will be discussed later. The study of fat formation in *T. lipofera* would also suggest that the efficiency of fat formation from carbohydrate may be influenced by temperature.

In this yeast, the conversion coefficient reaches its maximum between 20–25° (see Fig. 4).

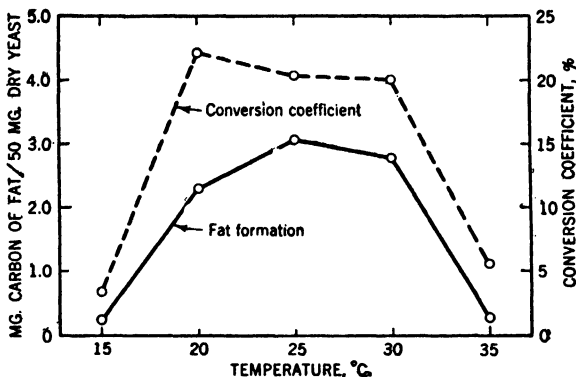


Fig. 4. Effect of temperature on fat formation and the conversion coefficient in *Torulopsis lipofera* (60).

3. pH

The optimal pH of the medium for fat formation depends greatly on the type of organism used. In many microorganisms growth and fat formation proceed best at a pH between 3.5 to 6. Higher or lower pH inhibits to some degree growth and fat formation. In *T. lipofera* the fat formation, as indicated by both R.Q. measurements and fat estimations, was highest at pH 5.5 to 6.0, and the conversion coefficient also decreased with increasing pH.

4. Phosphate

Phosphate appears to be essential for fat synthesis from carbohydrate. The role of phosphate in fat synthesis is not easy to assess, partly because, so far, experiments on fat synthesis can be carried out only with intact cells which have sufficient phosphate available for their metabolic processes, and partly because no experiments could be devised to separate the breakdown of carbohydrate, which may require phosphate, from the actual process of synthesis of fatty acids from the carbohydrate breakdown products. There is, however, some evidence to indicate that phosphate is required for the conversion of carbohydrate to fat. Smedley-Maclean and Hoffert (120,121) showed that in baker's yeast fat formation decreased in the

TABLE III
EFFECT OF PHOSPHATE ON FAT FORMATION IN *Torulopsis lipofera**

KH ₂ PO ₄ , M concentration	Glucose used, mg.	C of fat formed, mg.	Conversion coefficient, %
0	53.9	2.10	9.8
0.001	51.0	3.87	19.5
0.01	50.6	3.79	18.8
0.05	51.3	3.99	19.5

* 52.8 mg. yeast; pH 6.0; 25°; 5 hr.

absence of phosphate. A somewhat more detailed study of this effect was made in *T. lipofera*. In this yeast, phosphate in the medium is necessary for maximal synthesis of fat. It also appears from these experiments that the phosphate is required mainly for carbohydrate breakdown, since iodoacetate did not influence the conversion coefficient, but did decrease the amount of carbohydrate used and fat formed. Table III shows some of the results.

5. Various Factors (Age, Salts, Concentration of Substrate)

Under this heading falls a considerable number of factors which have been shown to influence fat formation in various microorganisms. Firstly, the *age of the cell* ought to be mentioned. In a number of organisms which normally contain only a small amount of fat (which forms part of the protoplasm, *i.e.*, *élément constant* of Belin, 10), large globules of fat may appear in aging cultures. This phenomenon points rather to a "degenerative fat formation," *i.e.*, synthesis of fat from other cell constituents under conditions injurious to the cell, than to a normal synthetic process in healthy cells. Such degenerative fat formation is also observed in old tissue cultures, or in tissue cultures which have been exposed to some toxic agent.

Figure 5 shows the relationship of fat content of *Penicillium javanicum* with the age of the culture (76). Similar results were also obtained by Nadson and Konokotina (89); see also Kordes (63).

That the *osmotic pressure of the medium* can play a role on the fat content was demonstrated by Halden (44), who partly dehydrated baker's yeast by placing thick suspensions of the yeast on porous tiles and maintaining it in a moist chamber, and found a considerable increase in the fat content of the cells under these conditions. Higher salt concentrations (sodium chloride, calcium chloride) appear to

bring about an increase of fat content in *Endomyces vernalis* rather by their osmotic effect than any specific action (48). Although in these experiments the percentage of fat in dry weight increased with increasing concentrations of sodium chloride (0–5%), the amount of mycelium decreased very steeply with increasing salt concentrations. The concentration of substrate may also play a role. Thus, in *Penicillium javanicum*, the fat content of the mycelium rises with increasing substrate concentrations (76). Similar observations were also made on other molds, e.g., *Aspergillus niger* (10), *A. fischeri* (99), and yeasts (118). Since in these experiments no clear-cut evidence for the effect of the concentration of substrate on the conversion of carbohydrate to fat was obtained, it is difficult to assess the real significance of these results.

The influence of various other salts on fat formation has been examined. Heide (48) states that sulfate ions are necessary for fat formation in *E. vernalis*. The optimal concentration is 0.037% (as sodium sulfate). Since his observations were made under conditions in which both growth and fat formation occur, his results cannot be taken as direct evidence that sulfate is required for fat synthesis. Magnesium and manganese

were not required in short-term experiments for the formation of fat in *T. lipofera*, because they did not increase the conversion coefficient (60). According to Macleod and Smedley-Maclean (77) magnesium and calcium decrease the fat formation from glucose in baker's yeast, and increasing concentrations of magnesium decrease growth and fat formation in *P. javanicum* (76). Lockwood *et al.* (76) examined also the effect of various trace elements on fat formation and state that some of them, e.g., columbium, molybdenum, tungsten, ferric ion, and chromic ion, slightly increase growth of the mold and also the percentage of fat in the dry weight. Porges (98) claims that zinc slightly increases fat formation in *A. niger*.

In spite of the wealth of experimental data on the effect of these factors on fat formation, very little clear-cut evidence for the influence of the above factors on the synthesis of fat from carbohydrate was obtained. Most of the experiments were carried out under con-

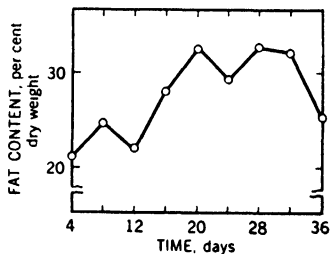


Fig. 5. Changes in fat content with age of culture in *Penicillium javanicum* (76).

ditions in which both growth and fat formation took place, thus preventing any definite conclusions. In addition, the cells contain, under normal conditions, most if not all of the necessary factors which may influence fat formation from carbohydrate, so that even when growth is excluded (resting cells) negative results of short-term experiments do not necessarily mean that the examined factor is not required for the process.

6. Vitamins

The effect of vitamins on fat synthesis has been reviewed by McHenry and Cornett (82) and only some pertinent data will therefore be given in this paper. A number of authors have shown that thiamine is essential for fat formation in the animal (81,83,84,148,149). In animals previously depleted of vitamins of the B group, the addition of thiamine to the diet caused a large increase in the fat of the animal, raised the R.Q. above 1.0, and at the end of the experimental period this fat contained a high proportion of palmitic and hexadecenoic acids (74), which, according to Longenecker (71), is characteristic of fat synthesis from carbohydrate. Other accessory food factors of the B group, *e.g.*, riboflavin, pyridoxine, pantothenic acid, and biotin, have also been shown to play a role in fat synthesis, augmenting the amount of fat formed in the presence of thiamine (85). Furthermore, in some microorganisms, *e.g.*, *Torula utilis*, thiamine has been shown to be of importance for fat synthesis (93). In *Torulopsis lipofera*, additions of thiamine had no effect on fat synthesis; this, however, does not exclude the possible role of this vitamin, since not inconsiderable amounts of thiamine accumulate in this yeast, and may be sufficient for fat synthesis in short-term experiments.

McHenry and collaborators also called attention to other dietary factors which might play a role in fat formation from carbohydrate, *e.g.*, vitamin A. For a detailed account and further references see McHenry and Cornett (82).

7. Effect of Assimilable Nitrogen and the Relationship of Fat Formation and Protein Synthesis

In the normal living cell the various metabolic processes are closely interlinked and finely balanced to meet the requirements of the cell. This balance can, however, be changed experimentally by altering

the relative amounts of the various nutrients for the cell. A supply of nutrients high in carbohydrate and low in protein (or nitrogenous substances which the cell is capable of using for protein synthesis) may therefore lead to an accumulation of fat in the cells. Thus, feeding animals a diet high in carbohydrate, but low in protein, leads to a rapid storage of newly formed fat (3,32,34). A similar result was obtained in plants. Schmalfuss (113) was able to show that the fat content in linseed was inversely related to the amount of nitrogenous fertilizer used. The most extensive study of this problem was made on microorganisms. It was shown by numerous authors that increasing the supply of nitrogen available for protein synthesis in the medium decreases the fat content of the cell, more carbohydrate being used for the growth of the cells. Further, under conditions in which no multiplication of the cells takes place, ammonium salts (or urea) decrease the efficiency of the conversion of carbohydrate to fat (see Fig. 6). In these experiments, however, protein synthesis without cell multiplication could not be excluded.

The relationship of protein and fat formation is of particular importance from the point of view of a possible application of the results obtained for the commercial production of fat by microorganisms, and accordingly considerable attention was paid to the problem. Since, in the presence of abundant nitrogen, microorganisms grow rapidly but form little fat, it is in practice possible to separate a growth phase from a fattening stage by regulation of the supply of nitrogen. This is the principle of the method evolved by Lindner and associates (see 38) for the cultivation of *Endomyces vernalis* as a fat-forming organism. These authors attempted to cultivate on a large scale the above organism on a medium containing sufficient nitrogen for rapid growth, and, after a thick mycelium was formed, substituted the medium for one containing only carbohydrate, thus inducing the organisms to form fat instead of protein. More recent work with this organism (48,100) extends the results of the previous workers. Thus Raaf obtained particularly high fat content of the cells by removing most of the nitrogen available in the medium by the use of Permutite, thereby changing the relation of nitrogen to carbohydrate in the substrate. In *Rhodotorula gracilis*, Enebo *et al.* (35) showed that good growth and high fat content of the cells can be obtained when the yeast is grown on a medium containing a convenient proportion of nitrogen to carbohydrate, allowing both rapid

growth, and, after using up the available nitrogen, good fat formation. These authors fix the optimal relation of nitrogen to glucose at about 0.5 g. nitrogen to 100 g. carbohydrate. Figure 7, taken from the work of these authors, demonstrates their results.

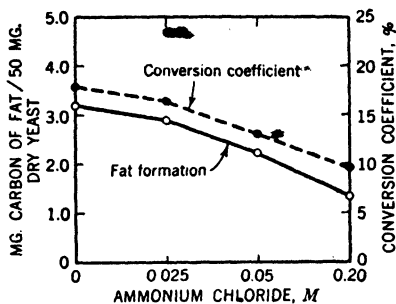


Fig. 6. Ammonium chloride effect on fat formation and the conversion coefficient in *T. lipofera* (60).

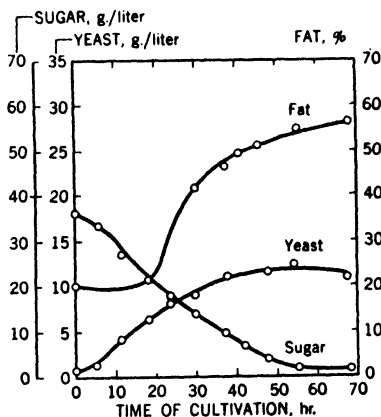


Fig. 7. Sugar content, amount of yeast, and percentage of fat as functions of time (35).

The inverse relationship of protein and fat synthesis in the living cell is possibly due to a competition of the enzymes concerned for the substrate, the velocity of amino acid synthesis being probably considerably higher than that of fatty acids. Raaf (100) arrived at the conclusion that probably both protein and fat arise from the same carbohydrate breakdown product.

D. SYNTHESIS OF FAT FROM VARIOUS CARBOHYDRATES

In the above account of fat synthesis the carbohydrate used was mainly glucose. There exists good evidence that the living cell can also employ some other carbohydrate for fat synthesis. Thus, Barber (6) found that a *Penicillium* grew and formed fat of similar chemical characteristics whether the mold was grown on glucose, sucrose, xylose, or glycerol. Lockwood *et al.* (76) studied growth and fat formation in *P. javanicum* on a number of different carbohydrates and found that only glucose and xylose gave good yields of mycelium and fat, xylose being utilized as readily as glucose. Other carbohy-

drates, *e.g.*, galactose, maltose, sucrose, starch, dextrin, glycerol, and mannitol, were definitely inferior to the above two carbohydrates. In *Endomyces vernalis*, Reichel and Reinmuth (103) state that fructose and sucrose are more easily converted to fat than glucose; Lindner (69), Nadson and Konokotina (89), and Heide (48) found that this microorganism can use a number of different carbohydrates (except pentoses) as carbon source for both growth and fat formation.

T. lipofera can equally well use glucose and fructose, and, if grown on sucrose, *i.e.*, when the cells had opportunity to adapt themselves to the new substrate, formed fat equally well on sucrose and glucose. Maltose could also be utilized for fat synthesis (Table IV).

TABLE IV
FORMATION OF FAT FROM VARIOUS CARBOHYDRATES IN *Torulopsis lipofera**

Expt. No.	Substrate	C of fat formed, mg.
1	Glucose	5.80
	Fructose	5.50
	Sucrose	3.68
	Maltose	3.38
2	Glucose	2.33
	Galactose	0.43
3	Glucose	2.12
	Sucrose	1.38
4	Glucose	2.81
	Sucrose	2.96

* Yeast in Expts. 1-3 grown on glucose; in Expt. 4, grown on sucrose. 50-52 mg. yeast; 25°; pH 5.5; 5 hr.

Recent experiments in the author's laboratory (61) show that yeast No. 72 can be adapted to utilize xylose for fat formation, while other pentoses, *e.g.*, ribose or arabinose, are not utilized. The experiments were carried out by measurement of the R.Q. using the Warburg technique. Table V summarizes the results. These experiments were also confirmed on a larger scale, when it was shown that xylose is equal to glucose as a carbon source for both growth and fat formation, the fat coefficient being in these experiments equal to that found with glucose.

Some other microorganisms, such as *Oidium lactis* (39) or *Fusarium* (27,88) can also use pentoses for fat formation.

TABLE V
ADAPTATION OF YEAST NO. 72 TO UTILIZATION OF XYLOSE*

Coeffi- cient	Yeast grown on glucose			Yeast grown on xylose		
	No substrate	Substrate		No substrate	Substrate	
		Glucose	Xylose		Glucose	Xylose
QO ₂	7.4	18.3	10.1	6.4	11.4	11.5
R.Q.	0.92	1.20	1.10	1.02	1.22	1.23

* Duration of experiments two hours.

The results obtained, especially those with pentoses, are of particular interest from the point of view of the possible mechanism of fatty acid synthesis from carbohydrate, and warrant a still closer examination. This aspect will be discussed at a later stage. As Bernhauer points out, the fact that pentoses can be used as a carbon source for fat formation may also be of importance for technical purposes.

E. SYNTHESIS OF FAT FROM VARIOUS NONCARBOHYDRATE SOURCES

Various substances known to be intermediates in carbohydrate metabolism have been examined as possible carbon sources for fat formation in the hope that the experimental results might help to elucidate the mechanism of fatty acid synthesis. It ought to be mentioned that great caution has to be used when examining the results, since experiments on intermediary metabolism with whole cells are more difficult to interpret than experiments with cell extracts. Even if no fat is formed from an examined substrate, this result does not exclude a possible role of the substance in question as an intermediate in fatty acid synthesis from carbohydrate. The permeability of the examined substrate into the cell under given experimental conditions may greatly influence the results. On the other hand, carbohydrate stored in the cell might be mobilized under some conditions for fat synthesis and the result might lead to false conclusions. The rapidly developing and new experimental technique, the use of isotopes, may help to overcome at least some of the difficulties.

The first to make a detailed study of fat formation from various carbon sources were Haehn and Kintoff (45). These authors allowed *Endomyces vernalis* to form a mycelium poor in fat on a carbohydrate

medium, replaced the medium by one containing only the substrate tested, and found that ethanol, acetaldehyde, glycerol, pyruvic acid, lactic acid, and aldol increased the amount of fat in the mycelium. Using glucose or ethanol as substrate the authors could demonstrate the formation of acetaldehyde as a metabolite (as the bisulfite compound) of this microorganism.

Fat formation from acetate was demonstrated in baker's yeast by Smedley-Maclean and associates (77,120,121), who also showed that the presence of calcium or magnesium ions decreases fat formation from acetate (Table VI). These authors also demonstrated acetaldehyde and pyruvic acid as metabolites during fat synthesis from glucose and acetate. It is of interest to note that in these experiments the carbohydrate content of the cells decreased during fat formation from acetate. Acetate increased the formation of fat from glucose in the timothy grass bacillus (130,131).

TABLE VI
FORMATION OF FAT FROM ACETATES IN BAKER'S YEAST

Solution	Increase in fat content, %
Water.....	41
Potassium acetate.....	180
Sodium acetate.....	160
Magnesium acetate.....	118
Calcium acetate.....	100

Recently, Rittenberg and Bloch (107), using the isotope technique, proved conclusively that acetic acid can be converted to fatty acids in animals (mice and rats). The saturated fatty acids contained a higher concentration of C₁₃ and D than the total fatty acids, and the concentration of isotopes was higher in the fatty acids of the liver than in those of the carcass. Utilization of acetic acid for fatty acid synthesis was also confirmed in yeast by the isotope technique (150), confirming also earlier experiments of Sonderhoff and Thomas (124), who used trideuterioacetic acid.

The formation of fat from ethanol was also demonstrated by Lindner (70) in *Endomyces vernalis* and Halden *et al.* (44,123) for baker's yeast. Smythe (122) demonstrated lipide formation from pyruvic acid in baker's yeast. Acetaldehyde was found to increase the fat

content of some microorganisms (*E. vernalis*, *T. lipofera*) but not of baker's yeast (121a).

F. SYNTHESIS OF FATTY ACIDS FROM PROTEIN

This, until recently controversial, question has been lately clearly answered by the work of Longenecker (71) and simultaneously by Hoagland and Snider (55). Longenecker showed that rats, which had been previously starved, rapidly formed fat when placed on a purified casein diet. The synthetic fat did not differ from that formed on a carbohydrate diet. The latter authors demonstrated fatty acid synthesis from protein by comparing the amount of body fat in animals at the beginning and the end of an experimental period during which the animals were kept on a pure protein diet (with the addition of yeast to supply the necessary accessory factors).

The work of McHenry and associates (see 82) suggests that some of the vitamins of the B group, especially pyridoxine, play a role in the conversion of protein to fat (see 41). The role of pyridoxine might be expected in the light of recent investigations which showed pyridoxine (or its derivatives) to be a coenzyme of some of the enzymes concerned with amino acid metabolism (68,112). Further work of McHenry would suggest that carbohydrate is not formed as an intermediate in fat formation from protein.

It ought to be mentioned that in microorganisms synthesis of fatty acids from protein has not yet been demonstrated conclusively.

G. INTERCONVERTIBILITY OF FATTY ACIDS

Schoenheimer and associates (114) have shown that the animal is capable of rapidly lengthening or shortening the chain length of fatty acids. Thus, deuterium-enriched palmitic acid supplied to the animal is rapidly converted to stearic and also to myristic (lauric) acids (134). The shortening of the chain length might proceed according to the suggested schemes of the degradation of fatty acids in carbon pairs. Furthermore, since deuterium was also found in unsaturated fatty acids, *e.g.*, oleic and hexadecenoic acids, and, on the other hand, it was shown that deuterium-enriched oleic acid can be converted to stearic acid, it appears that the animal is capable of synthesizing most of the component fatty acids from one fatty acid.

While the enzymes necessary for the observed desaturation of higher fatty acids are well known (for reviews, see 79,127), enzymes

from animal tissues which could bring about the saturation of, for example, oleic acid to stearic acid have not yet been described. To the author's knowledge there exists in the literature only one claim that such a hydrogenase (saturase) of higher fatty acids exists. Zeller and Maschek (151) found that when an emulsion of oleic acid was added to an extract of marrow seeds, the oleic acid disappeared (measured by uptake of iodine). Mull and Nord (88) explain their results (*Fusarium* acting on oleic acid brings about subsequent decreases and increases of the iodine absorption) by oxidation of the substrate with the formation of fatty acids of lower chain length rather than by assumption of a saturation of oleic acid.

The above account might appear to explain sufficiently the mechanism of formation of most fatty acids in the animal body from one or few "primary" fatty acids. However, as pointed out by Hilditch (50), the constitution of some minor component fatty acids present in animal fat, *i.e.*, the position of their double bonds, makes it difficult to accept such an assumption. Thus " β -oxidation" of oleic acid would be expected to lead to Δ^7 -hexadecenoic and Δ^5 -tetradecenoic acids, while these acids found in animal fats have their ethylenic linkages in the Δ^9 -position (as does oleic acid).

H. SYNTHESIS OF GLYCERIDES FROM FATTY ACIDS

When speaking about the formation of fat from carbohydrate, the main attention was focused on the synthesis of fatty acids. Animal fat usually contains only small amounts of free fatty acids, and one has to assume that the newly synthesized fatty acids are immediately esterified with glycerol (formed by the breakdown of carbohydrate according to the well-known mechanism) to triglycerides. In cells of other origin, *i.e.*, microorganisms or plants, this statement does not necessarily hold true. The fats isolated from many microorganisms contain large amounts of free fatty acids. The rather drastic methods often used in isolating the fat from these organisms (*e.g.*, extraction with acidified fat solvents, or acid hydrolysis of the material prior to extraction) make it occasionally difficult to decide whether the observations were not artifacts. In some instances, however, there exists clear indication that a considerable proportion of the fatty acids is free. In linseed during fat formation the high proportion of free fatty acids found at first (in some varieties as high as 42%) gradually decreased with the maturing of the seed (8), suggest-

ing that the formation of fatty acids proceeded independently of the formation of glycerides (see also 56).

I. SITE OF FAT FORMATION

Heide (48) studied the cytology of *Endomyces vernalis* and arrived at the conclusion that the fat formation is closely connected with the metabolic processes in the vacuoles of the cells. The first visible droplets of fat were formed on the border of the vacuole, and during further fat synthesis these droplets enlarge and fuse, so that eventually a large globule fills most of the cell. Parallel with the increase of fat the vacuoles diminish in size.

In animals the problem of the site of fat formation is more difficult to determine because, in addition to fat synthesis, transport of fat from one tissue to the other, and also deposition of dietary fat take place. The use of the isotope technique has, especially in Schoenheimer's laboratory, helped to answer some of the problems. Thus Sperry *et al.* (126) fed rats with fat containing stably bound deuterium and showed that deuterium-containing fatty acids were rapidly deposited in the liver, skin, and intestine of the animal, while only traces of deuterium-containing acids were found in the nervous tissue (see also 80). When, however, animals were fed with water enriched with D_2O , deuterium was incorporated as rapidly in the brain as in the depot fat (142), and, during maximum myelinization in rats lipides were synthesized in the brain and nerves even more rapidly than in any other tissue (143). From these experiments, and especially from the rate of incorporation of body fluid deuterium into fatty acids in various tissues, it would follow that animal fat is synthesized in at least the following tissues: liver, skin, intestine, and brain. Furthermore, it appears that the nervous tissue does not use dietary acids, but synthesizes its own fatty acids. Quantitatively, the liver would seem to be the main site of fat synthesis in the animal body. In spite of the peculiar composition of milk fat, which differs widely from that of body fat, it does appear that the milk gland is not a site of fat synthesis, since most of the milk fat could be accounted for from the uptake of fat from the blood by the active mammary gland (116a).

III. Composition of Fat and Factors Influencing It

This paper would not be complete without reference to the composition of the fat formed. It is not proposed to give details of the

composition of fats of various origins, since this forms the subject of a number of excellent monographs, such as the book of Hilditch (50). It may, therefore, be sufficient to recall that the composition of depot fat in the animal kingdom shows, in addition to species specificity, a definite phylogenetic specificity, *i.e.*, the relative amounts of saturated and unsaturated fatty acids, and also the relationship of individual fatty acids changes characteristically when comparing the various stages of phylogenetic development. Table VII makes this clear (53).

TABLE VII
PERCENTAGE WEIGHT OF COMPONENT ACIDS IN ANIMAL DEPOT FATS

Animal	Saturated Palmitic	Unsaturated			
		C ₁₆	C ₁₈	C ₂₀	C ₂₂
Fish, fresh water	13-15	ca. 20	40-45	ca. 12	0-5
Marine	12-15	15-18	27-30	20-25	18-12
Whale	12-15	15-18	35-40	15-20	5-10
Frog	11	15	52		15
Tortoise	14	9	65		7
Lizard	18	10	56		5
Domestic fowl	25-26	6-7	ca. 60	0.5-1	
Rat	24-28	7-8	ca. 60	0.3-0.5	
Kangaroo	25	3	48		3
Cat	29	4	43	Trace	
Pig	25-29	2-3	50-65	0.3-1	
Ox	27-30	2-3	40-50	0.2-0.5	
Sheep	23-28	1-2	40-50		0.6
Bear (sloth)	29	11	52		2
Lion	29	2	40		3
Baboon	19	4	67		0.5
Human	24-25	5-7	53-57		2-2.5

A detailed study of fat from various organs also showed characteristic differences in the relative amount of the component fatty acids. Table VIII shows a comparison of the content of component fatty acids in the lipides of the depot and the liver (50). It should be added, that the component fatty acids of milk fat and of some lipides of the brain (*e.g.*, cerebrosides) are characteristic. Thus milk fat contains a high proportion of fatty acids of short chain length (C₄-C₁₀). The cerebrosides of the brain contain normal C₂₂, C₂₄, and C₂₆ fatty acids and their α -hydroxy derivatives (25).

While the component fatty acids of animal depot fat do not show wide variations in the number of acids usually found, plant fats differ considerably in the type and constitution of component fatty acids, and hitherto unknown fatty acids are continuously being discovered.

From the point of view of constitution, the finding (22) of a C_{18} fatty acid containing one terminal double bond and two acetylenic link-

TABLE VIII
DISTRIBUTION OF INDIVIDUAL FATTY ACIDS IN LIPIDES FROM SOME ORGANS

Acid	Depot glycerides	Liver glycerides	Liver phospholipide
Palmitic	High	High	Lower
Stearic	High	Low	High
Hexadecenoic	Very low	Higher	Medium
C_{18} unsaturated	High	High	Lower
C_{20} and C_{22} unsaturated	Very low	Medium	High

TABLE IX
ANALYSIS OF YEAST FAT

Analysis	Newman and Anderson (91)	Täufel et al. (135)	Peck and Hauser (96)	Reichert (105)	Hilditch (51)
	<i>Saccharomyces cerevisiae</i>		<i>Blastomyces*</i>	<i>Torula utilis</i>	Yeast No. 72
Fat content (per cent dry wt.)	6.9	—	9.5	6.4	30 (av.)
Chemical characteristics					
Saponification number	109.6	156.6	191.5	180.7	205.5
Acid number	28.6	108.4	45.3	102.4	67.8†
Iodine value	61.3	130.4	106.1	120.5	62.6
Unsaponifiable matter, %	46.7	19.6	8.0	12.3	2.4
Fatty acids, %	47.4	66.4	88.0	77.3	
Component fatty acids					
Steam volatile		7.3			
Myristic				0.3	0.1
Palmitic	ca. 13.5	13.5	11.3	7.9	25.6
Stearic	ca. 4.5	8.3	5.5	3.8	5.9
Saturated C_{20} , C_{22} , and/or higher				0.2	5.1
Liquid-saturated fatty acids				4.6	
Hexadecenoic	ca. 19.5			7.6	1.3
Oleic	58.5	66.9	62.5	21.5	54.5
Octadecadienoic		4.1	21.0	49.7	5.7
Octadecatrenoic				4.4	0.7
Unsaturated C_{20-22}					1.1
Unidentified unsaturated	9.9				

* *B. dermatitidis*. Results of one analysis of a series of two.

† The high acid value of this yeast fat is, in the author's opinion, due to the procedure used in isolating the fat. Hydrolysis of the yeast with 1.0 N hydrochloric acid prior to extraction of the fat usually gives a fat with only 2-5% free acids (calculated as oleic).

ages (erythrogonic acid) is of particular interest. Much attention is being paid to the component fatty acids of acid-fast bacteria, many of which contain branched chains (see 1). Branched-chain fatty acids have recently also been isolated from wool fat (145).

Since much of the work described in previous chapters has been done on yeast, some data on the composition of yeast fat will be given, since this subject has recently received considerable attention. In Table IX are recorded some of the analyses.

It will be seen from this table that the fat from the only typical fat yeast (No. 72) is characterized by a low content of unsaponifiable material as compared with the other yeasts. The component fatty acid represent a relatively simple mixture. No such detailed analyses have been carried out so far on the fat of other fat yeasts. The chemical characteristics of the fatty acids isolated from some other fat yeasts (*Torulopsis lipofera*, yeast No. 74) in the writer's laboratory differed only in the iodine value, as shown in Table X (61). It will be shown later that the degree of unsaturation of fatty acids of these yeasts is dependent to some extent on the conditions of cultivation.

According to some analyses of yeast fats, volatile fatty acids were found among the component fatty acids (see Table IX). Weiss (144a) actually isolated an optically active, steam-volatile acid, most probably a mixture of two isomeric isovaleric acids. In these analyses the methods of cultivation of the yeast used and the conditions of isolation of the fat were not reported. In the author's opinion, where commercial samples of yeast or yeast fat are used, it might not always be possible to exclude an admixture to the fat of various antifoaming

TABLE X
ANALYSIS OF FAT FROM VARIOUS FAT YEASTS*

Analysis	<i>Torulopsis lipofera</i>	Yeast No. 72	Yeast No. 74
Unsaponifiable matter, %	2.64	1.74	0.67
Fatty acids, %	92.1	92.4	93.7
Mixed fatty acids, I.V.	73.6	61.0	52.7
Saturated fatty acids (Twitchell), %	19.96	30.65	38.80
Iodine value	0.82	4.62	0.89
Saponification equivalent	273.7	273.0	272.5
Mixed saturated fatty acids, m. p.	—	54° C.	54° C.
Unsaturated fatty acids, %	80.04	69.35	61.20
Iodine value	92.5	91.2	88.9
Saponification equivalent	290.5	283.4	281.5

* The fat was isolated from the residue after acid hydrolysis of the yeast.

agents used in the cultivation, thus influencing the analyses of the component fatty acids.

The composition of fat can be considerably influenced by a number of factors. This is to be expected when it is realized that fats are not inactive reserve material, but take active part in the metabolism of the cell or organisms, as was shown by the work of Schoenheimer and associates (114), *i.e.*, at the same time fats are continuously being utilized and synthesized. Changes in the metabolism of the living cell may therefore bring about changes in its fats.

In animals, the most important factor influencing the composition of depot fat is the nature of the diet. The deposition of dietary fatty acids in the depot fat of the animal can change to a considerable extent the composition of the fat. This has been demonstrated by numerous investigators (see reviews of Anderson and Williams, 4, and Mendel and Anderson, 86). Thus, Ellis *et al.* (33) showed that increasing amounts of cottonseed oil (major component fatty acids: palmitic, oleic, and linoleic acids) added to a standard diet bring about considerable changes in the depot fat of pigs (Table XI).

TABLE XI
DEPOT FAT OF HOGS FED VARYING RATIOMS OF COTTONSEED OIL (33)

Diet	Fat, I.V.	Component fatty acids, %				
		Myris- tic	Palmi- tic	Stearic	Oleic	Lino- leic
Basal diet	60.6	1.7	25.5	13.7	50.2	8.9
and 4% oil	60.5	1.1	25.0	21.1	39.5	13.3
and 8% oil	64.4	0.8	21.9	23.3	35.8	18.2
and 12% oil	77.4	1.1	13.8	26.5	31.8	26.8

When, on the other hand, the animal is placed on a diet high in carbohydrate and low in fat, it rapidly synthesizes fat of a characteristic composition. Thus Longenecker (71,72,74) demonstrated that rats on a high-carbohydrate, fat-free diet deposited fat, which contained a particularly large amount of C₁₆ acids (palmitic and hexadecenoic acids). Similar results were also obtained when fat formation from carbohydrate was enhanced by the addition of thiamine to the diet (74). This result is in agreement with earlier work, especially of Ellis and associates (32,34), who demonstrated the hardening effect of a diet high in carbohydrate on the depot fat of pigs. The

importance of these observations on the views on the mechanism of fatty acid synthesis will be discussed later.

In fasting animals (rats) the composition of the fat undergoes but slight changes (71); similar results were also obtained in pigs (54).

Under normal dietary conditions animals appear to have a tendency of maintaining the relationship of saturated and unsaturated fatty acids constant within certain limits (threshold level, see 7,71, 75).

Temperature, in animals, appears to influence the unsaturation of the depot fat. When comparing the setting points and iodine value of depot fat of pigs taken from different layers of the body surface (or maintained for some time at different temperatures) Henriques and Hansen (49) showed that a definite relation exists between the temperature of the tissues and the saturation of the fat, *i.e.*, the higher the tissue temperature, the higher the setting point, and the lower the iodine value (I.V.), of the fat. Thus, perinephric fat had an I.V. of 47.7 compared with 60 of the outer-back fat. These results were later confirmed by Dean and Hilditch (29). Since the melting points of fats of cold-blooded animals are lower than those of birds and mammals, a generalization of the above-stated relation of temperature and saturation of fats might be assumed. Hammond (47) explains this by the assumption that fats have to be liquid in the tissue in order to be of use as a source of energy to the animal. Hilditch (50) warns that no such wide generalization can be drawn between the tissue temperature of the organism and the composition of its fat, pointing out that, for instance, the composition of the fat of marine mammals resembles very much that of fishes. However, since dietary fat can greatly influence the composition of depot fat (as shown above) one might expect that the composition of fat of, *e.g.*, the whale will resemble to some extent the fat of the organisms it uses for nutrition.

Fraenkel and Hopf (41a) studied the effect of breeding temperature of insects on the iodine value of phospholipides. Thus in *Phormia* the I.V. of the isolated phospholipides dropped from about 90 (at a breeding temperature of 18°C.) to about 64 at a breeding temperature of 36°C. Similar results were obtained in the species *Calliphora*.

The effect of temperature has also been studied in some micro-organisms. In some molds, Pearson and Raper (95) and also Terroine *et al.* (140) found that the I.V. of the fatty acids decreased with

increasing temperature of cultivation, while in *Aspergillus fischeri* no effect of temperature was found (99).

The *pH* of the medium also appears to influence the composition of fat, as shown by Pontillon (97), who found that the melting point of fatty acids of *Aspergillus niger* was somewhat higher when the mold was grown on acid medium than on neutral or slightly alkaline medium.

A number of authors found that the concentration of substrate (glucose) influences the degree of saturation of the fat. Thus, in *A. flavus* (144), the melting point of the fat increased when the concentration of substrate was raised from 20 to 30–40%. For further references on this subject, see Bernhauer (13).

One may therefore summarize the foregoing by stating that the composition of fat in the living cell, in itself specific, may be influenced by changes in the metabolism (temperature, *pH*, age, etc.) and also by nutritional factors (fattening or starvation, composition of food, component dietary fatty acids, and also their possible preferential absorption).

IV. Mechanism of Fatty Acid Synthesis

Longenecker (73) in his review summarizes the criteria for any proposed mechanism of fatty acid synthesis in the living cell as follows:

In order to prove completely satisfactory, any proposed mechanism . . . should be in accord with the following:

(1) The qualitative and quantitative fatty acid composition of the "synthetic" fat.

(2) The position of the double bond in the unsaturated acids synthesized, and the *cis* configuration of these acids.

(3) The *in vivo* relationship of the vitamin B complex to the synthesis.

(4) A rate of synthesis comparable with that observed *in vivo*.

(5) Equal rates of synthesis for the saturated acids and somewhat lower rates of synthesis for the unsaturated acids. (This point may require some modification after further studies have been performed.)

(6) Approximately one-half of the hydrogen atoms should be derived from the body water and the other half should remain attached to the carbon chains undergoing transformation.

(7) The hydrogen derived from body water must be evenly distributed throughout the molecule.

In the foregoing a short account of the conditions of fatty acid (fat) synthesis and of the composition of fat has been given. It remains now (1) to summarize the evidence available for the possible nature of

the first product (products) of synthesis in the biological conversion of carbohydrate to fat; and (2) to review the existing theories on the mechanism of fatty acid synthesis from carbohydrate in the light of available evidence.

A. POSSIBLE NATURE OF THE FIRST PRODUCT OF SYNTHESIS

In the last few years considerable evidence has accumulated to show that rapid fat synthesis in animals or some microorganisms leads to the formation of fat of a rather saturated character. Some of the experimental results have already been mentioned previously. The most detailed study was made by Longenecker (71,72,74), who showed that the major fatty acids in "synthetic fat" were palmitic, oleic, and hexadecenoic acids. Hilditch and associates (5,52) concluded that animal fats synthesized from carbohydrate are predominantly palmitodi- C_{18} -glycerides. A decrease in the unsaturation of the fat during fat synthesis was also observed by Mendel and Anderson in pigs (2), and by Kleinzeller in *T. lipofera* (60). Some unpublished experiments from the author's laboratory give still further confirmation to these observations. As shown in Table XII, in three strains of fat yeasts during rapid fat formation from carbohydrate the I.V. decreases with increasing fat content of the cells. Boxer

TABLE XII
CHANGES IN IODINE VALUES OF FATTY ACIDS IN YEAST FAT
DURING FAT FORMATION (61)*

<i>Torulopsis lipofera</i>		Yeast No. 72		Yeast No. 74	
Per cent fat, dry wt.	I.V.	Per cent fat, dry wt.	I.V.	Per cent fat dry wt.	I.V.
20.4	90.5	27.9	62.7	17.7	59.6
19.3	93.5	32.5	59.3	24.6	48.2
24.1	82.3	—	—	32.4	49.1
25.5	80.5	—	—	40.5	43.1

* Samples from the same culture withdrawn at various stages of fat formation and analyzed.

and Stetten (17) also demonstrated that during fat synthesis in animals supplied with D_2O the saturated fatty acids contained a higher percentage of deuterium than the unsaturated ones.

Furthermore, the observation of Rittenberg and Bloch (107)—that during fat formation from isotope-labeled acetic acid (C_{13} in the carboxyl group and D in the methyl group) the saturated fatty acids

contained a considerably higher amount of both isotopes than the unsaturated acids—would also suggest that fatty acids synthesized from acetic acid are of a saturated character. Further work of these authors (108) showed that the distribution of isotopes was even throughout the molecules of fatty acids formed, giving convincing proof for a condensation of C_2 units to form the carbon chain of fatty acids.

On the other hand, the inability of mammals to synthesize some more highly unsaturated fatty acids (*e.g.*, octadecadienoic and octadecatrienoic acids; see 18,19,134) would appear to suggest that these

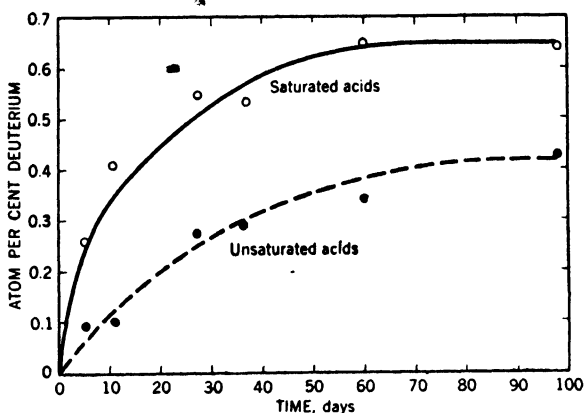


Fig. 8. Deuterium content of saturated and unsaturated fatty acids (109).

compounds are not intermediates in the synthesis of fatty acids from carbohydrate. Highly unsaturated compounds have been postulated to be intermediates in fatty acid synthesis by some theories, and Reichel and Schmid (104) actually claimed that they demonstrated the formation of fatty acids from hexadienal and octatrienal (which might arise by condensation of several molecules of acetaldehyde) in *Endomyces vernalis*.

The work of Schoenheimer and associates also showed that in the synthesis of fatty acids a considerable proportion (about 50%) of the hydrogen atoms of the acids was derived from body water (109). The rate of synthesis of the saturated fatty acids (palmitic and stearic) was about twice that of the unsaturated acids (see Fig. 8).

In some plant seeds (*e.g.*, linseed) the first product of synthesis does not appear necessarily to be a saturated fatty acid. In the experiments quoted earlier (37; see Fig. 2, page 301) the iodine value of the linseed oil was 114–127 up to the seventeenth day after flowering, *i.e.*, until the percentage of oil content in the seeds had about reached its maximum, and then the I.V. rapidly increased further to reach the final value of 190.

The experimental results quoted above appear to suggest that the fatty acids synthesized from carbohydrate (or C_2 compounds) are saturated acids. The available evidence does not allow us to state whether the unsaturated fatty acids are formed by secondary dehydrogenations of the saturated fatty acids (and this could only be limited to some unsaturated fatty acids) or whether a different pathway for the synthesis of the unsaturated acids exists.

According to some views on the mechanism of fatty acid synthesis, long-chain aldehydes are considered to be intermediates in the synthesis. In this connection the work of Waelsch and collaborators should be mentioned. It was shown (142) that in rats fed D_2O the concentration of deuterium was particularly high in the alcohol fraction of the unsaponifiable material, while very little deuterium was found in the higher fatty aldehydes (31). The authors discuss the possibility that higher fatty alcohols may be intermediates in the formation of fatty acids from carbohydrate.

B. INTERMEDIATE STAGES BETWEEN CARBOHYDRATE AND FATTY ACIDS

Two major theories have been put forward to explain the mechanism of fatty acid synthesis from carbohydrate: (1) Emil Fischer suggested condensation of carbohydrate molecules with the formation of long chains, and subsequent reduction of the hydroxyl groups. (2) The carbohydrate is first broken down to C_2 (or C_3) compounds, which then condense to form long chains. Among these compounds especially acetic acid, acetaldehyde, and pyruvic acid were considered. Since both these views (and their modifications) have been fully discussed in a number of monographs, it may be unnecessary to repeat them in detail. For detailed accounts see Smedley-Maclean (119), Bernhauer (13), and Hilditch (50).

The balance of evidence at the present time appears to be in favor of the breakdown of carbohydrate prior to a condensation.

Evidence: (1) Synthesis of fatty acids from C_2 compounds (ethanol, acetaldehyde, acetic acid) and C_3 compounds. It might be argued that these compounds are first condensed to carbohydrate, and only then condensed to fatty acids. This argument cannot be valid in view of the work of Rittenberg and Bloch (107) and also White and Werkman (150). These authors demonstrated that the carbohydrate of the tissues or of the yeast did not contain any appreciable amounts of isotopes when labeled acetate was used as substrate for fat synthesis. No information is, however available as to the mechanism of this condensation. Although condensation reactions of, e.g., acetic acid are well known (formation of acetoacetic acid—reaction by which acetic acid enters the tricarboxylic acid cycle), details of their mechanism have not yet been established. (2) Synthesis of fatty acid from pentoses. This might be expected to lead to the formation of at least some fatty acids with an odd number of carbon atoms, which

TABLE XIII
PHOSPHORUS DISTRIBUTION IN *Torulopsis lipofera*

Medium	Acid-soluble P, Mg./g. yeast as					Total
	ortho-phosphate	pyrophosphate	organic P	hexose diphosphate	alkalilabile P	
Glucose, 0.002 M KH_2PO_4	1.15	2.04	2.14	1.75	0.30	5.27
Glucose, 0.002 M KH_2PO_4 , 0.0003 M iodoacetate	0.85	2.06	3.26	2.68	0.14	6.17

were not found in nature. In this connection it should be recalled that fat formed from pentose did not differ in its chemical characteristics from that synthesized from glucose (6). A more thorough investigation of the metabolism of pentose in a fat yeast, now being carried out in the author's laboratory, might shed some further light on this subject. (3) Accumulation of phosphorylated intermediates of carbohydrate breakdown. Under conditions in which fat formation is only partially inhibited, iodoacetate leads to an accumulation of phosphorylated intermediates of carbohydrate metabolism in the yeast cell (60); see Table XIII. All the above arguments may be valid only if it is assumed that only one pathway of fatty acid synthesis exists.

The sole argument cited in favor of Fischer's theory is the fact that C_{18} acids predominate in the component fatty acids found in nature, and that, in addition to these, fatty acids containing a multiple of six carbon atoms appear to be most frequent (C_{12} and C_{24}).

None of the existing views can, however, explain satisfactorily the occurrence of specific groupings most frequently found in fatty acids, *e.g.*, $RCH=CH(CH_2)_7COOH$. It was mentioned above that in animal tissues dehydrogenases have been found which have been stated to dehydrogenate specifically stearic acid to oleic without further attacking the oleic acid formed. This finding would agree with the fact that the animal organism is not capable of synthesizing more highly unsaturated derivatives of oleic acid, *e.g.*, octadecadienoic and octadecatrienoic acids. The formation of the last-named acids in lower animals and in plants might possibly proceed with the help of some specific enzyme capable of further dehydrogenating oleic acid. This, however, is speculative, since there is no evidence either for the synthesis of the more highly unsaturated fatty acids from oleic acid, or for their formation from carbohydrate by a specific pathway. Actually, the new interpretation of the earlier-described observations on the increase of the I.V. in maturing seeds, offered by Chibnall (see 50), suggests that the formation of the more highly unsaturated fatty acids might proceed in these seeds independently of the formation of, *e.g.*, oleic acid.

No suggestions have been offered as to the mechanism of synthesis of some structural peculiarities of fatty acids, *e.g.*, the *cis* configuration of unsaturated acids, branched chain acids, or those containing one or several acetylenic linkages. It should also be mentioned that the conditions governing the formation of the chain length of fatty acids particular for a given tissue, or species, are not clear either.

It would appear that the available evidence does not allow a statement to be made as to whether fatty acids found in nature have all been synthesized by one mechanism, with subsequent metabolic changes of the first product of condensation (lengthening or shortening of the chain length, desaturation, or saturation, etc.), or whether several pathways of synthesis of fatty acids of major or minor importance exist. The author considers that a clear-cut statement as to the conditions of biological conversion of carbohydrate to fatty acids is at present of more value than any speculation about possible mechanisms without solid experimental evidence supporting it.

It may therefore be possible to summarize: (1) Biological synthesis of fatty acids from carbohydrate is an aerobic process, accompanied by a production of carbon dioxide. (2) Phosphate enhances fat formation from carbohydrate. The available evidence does not make certain whether phosphate is required only for carbohydrate breakdown or also for the condensation of carbohydrate breakdown products into fatty acids. (3) Vitamins of the B group, especially thiamine and pyridoxine, play a role in the formation of fatty acid from carbohydrate (and protein). (4) Fatty acids can be synthesized from a number of C_2 and C_3 compounds. The carbon of C_2 compounds used as substrate is evenly distributed throughout the chain length of the synthesized fatty acid. (5) Body water supplies about 50% of the hydrogen atoms of the fatty acids synthesized. The hydrogen is evenly distributed throughout the chain length. (6) The saturated fatty acids are synthesized from either carbohydrate or carbohydrate breakdown products more rapidly than unsaturated acids.

V. Synthesis of Phospholipides

This subject has lately been reviewed by Sinclair (117) and it is therefore proposed to deal with it only briefly.

The synthesis of phospholipide in the animal was demonstrated by the use of labeled fatty acids or radioactive phosphorus. The turnover of these labeled compounds suggests that in the animal the liver is the main site of synthesis of phospholipides.

In microorganisms phospholipides usually form a considerable proportion of the cell lipides. Thus, yeast phospholipides have been stated to form 13% (91), 26.8% (28), and 60–80% (30) of the total lipides. In microorganisms the synthesis of phospholipide from carbohydrate is evident, since these organisms can be cultivated on synthetic media. Using radioactive phosphorus, Chaikoff *et al.* (40,136) also demonstrated synthesis in liver slices. Their further work is particularly interesting because it sheds some light on the metabolic processes taking place during phospholipide formation. Incubating liver slices in a medium containing P_{32} , the incorporation of phosphorus into phospholipide was demonstrated under aerobic conditions, while under anaerobic conditions no phospholipide was formed. This is in agreement with calculations showing that the incorporation of phosphoric acid into phospholipide is an energy-consuming reaction, and therefore synthesis of phospholipide must be coupled with

an energy-producing reaction. Further experiments showed that various respiratory inhibitors such as cyanide, azide, hydrogen sulfide, and also carbon monoxide inhibit phospholipid synthesis, suggesting the participation of the cytochrome system in this process.

The exact mechanism and the sequence of the various synthetic processes involved in phospholipid synthesis (esterification of phosphate with glycerol or diglyceride, esterification with the amino moiety) are still obscure.

VI. Synthesis of Sterols

The synthesis of sterols has been clearly demonstrated by a number of authors (23,101,115), and it was shown by Schoenheimer and associates (15,16) that the animal can utilize acetic acid as substrate for sterol synthesis. At the same time, the experiments from Schoenheimer's laboratory showed that deuterium from body fluid is used in the synthesis of cholesterol (109).

In microorganisms (molds and yeasts) sterol (mainly ergosterol) forms a high proportion of the unsaponifiable material. The amount of sterol in these organisms varies between 0.1 and 1.7% (for references, see 13). Since these organisms (*e.g.*, yeast) can grow on synthetic media, the formation of sterols from carbohydrate substrate is evident.

The conditions of formation of ergosterol have been studied in some detail in yeasts and molds. Synthesis of sterols from carbohydrate is an aerobic process, since in nonaerated suspensions, or under anaerobic conditions, no sterols were formed in yeast, while with good aeration the amount of ergosterol increased from 2.65 to 8.4–12.9 mg. per g. yeast (dry weight) (78). Although pH and salts influence the formation of sterols (9,97), no evidence is available which would allow conclusions to be drawn as to the exact dependence of sterol synthesis upon these factors. There exists, however, good evidence that ergosterol can be synthesized from some C₂ compounds. Thus Halden *et al.* (44,123) succeeded in increasing the sterol content about sixty times by keeping a yeast suspension in thin layers on agar containing sucrose, and supplying oxygen and alcohol vapors. Sonderhoff and Thomas (124) demonstrated that when using trideuterioacetic acid as substrate, a considerable amount of deuterium accumulated in the unsaponifiable material, suggesting a synthesis of sterol from acetate. Further evidence for the synthesis

of sterol from acetate was supplied in yeast (78). It is of interest to note that most of the sterols formed were present as esters (78), and the idea was advanced that sterols might act as a detoxicating agent against the excessive production of fatty acids. The validity of this view appears to be questionable since in resting cells of certain other yeasts rapid fatty acid synthesis occurs without appreciable formation of unsaponifiable material (see Table II).

Although there now exists some information about the possible short-chain compounds which may serve as basic units for the synthesis of sterols, the mechanism of this condensation is still completely obscure.

VII. Synthesis of Some Wax Constituents

A number of investigators examined the metabolism of plant and insect waxes. The constituents of some characteristic waxes from insects and higher plants are listed in Table XIV.

Hall and Gisvold (46) found that the wax isolated from growing tips of *Pinus caribea* contained, among others: cyclic components; paraffins C_6-C_9 ; *n*-saturated fatty acids C_{16} , C_{20} , C_{30} ; melissyl alcohol (*n*-1-triacontanol); 10-nonacosanol; and probably unsaturated C_{18} acids. The waxes of marine animals are characterized by shorter chain aliphatic alcohols of twelve to twenty carbon atoms, and also unsaturated aliphatic alcohols $C_{16}-C_{22}$. In addition to these alcohols in ester linkage with fatty acids, some of these alcohols, *i.e.*, hexadecanol, octadecanol, and octadecenol, are found in waxes of some marine fishes in ether linkage with glycerol, and are known under the names of chimyl, batyl, and selachyl alcohols, respectively. The saponifiable material from marine animals and also from some microorganisms contains an unsaturated hydrocarbon, squalene, $C_{30}H_{50}$, and also paraffins isooctadecane (pristane) and possibly octadecene. For details on the composition of plant and animal waxes, see Hilditch (50). For details on the constitution of waxes of acid-fast bacteria the authoritative review of Anderson (1) should be consulted.

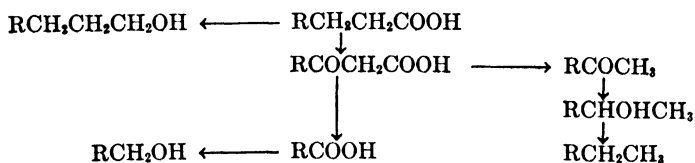
Evidence for the synthesis of waxes from other constituents of plants was supplied by Sahai and Chibnall (111), who showed that while no wax was present in the seeds of Brussels sprout, the amount of wax in the leaves of the seedlings increased with the age of the plant (Table XV). Similar results were obtained on examining the runner bean (57). Rapid synthesis of waxes is obvious in micro-

organisms especially in acid-fast bacteria, because of the high content of waxlike substances in these bacteria (5–11% of dry weight) when grown on purely synthetic media.

TABLE XV
AMOUNT OF WAX IN LEAVES OF BRUSSELS SPROUT OF VARIOUS AGES

Age above ground, days	Condition of plants	Per cent of fresh weight		
		Total ether extract	Wax	Glyceride fatty acids
	Ungerminated seed	37.8	0.0	33.8
10	Seedlings 2 cm. high	0.64	0.05	0.17
22	Seedlings 3–5 cm. high	0.83	0.10	0.16
43	Plant 8–10 cm. high	0.74	0.13	0.15
54	Plant, laminae 8–10 cm. long	0.75	0.15	0.14
126	Mature plants, laminae 15–25 cm. long	0.49	0.18	0.09
221	Aged plants	—	0.21	0.11

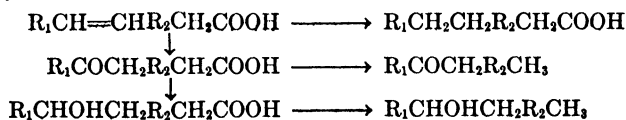
On the basis of their investigations Chibnall and Piper (24) suggested a scheme of synthesis of wax constituents (higher aliphatic alcohols, ketones, and paraffins) from fatty acids. The authors assume that the primary alcohols found in waxes are formed by reduction of the respective fatty acids. Oxidation on the β carbon, or condensation with a C_2 compound would then lead to the formation of alcohols of shorter or longer chain length, as shown in the following scheme:



It is of interest to note that in the animal (rat) Stetten and Schoenheimer (134) were able to demonstrate that higher alcohols (*e.g.*, hexadecanol and octadecanol) are readily converted to the respective fatty acids (palmitic and stearic acid) and vice versa. According to the above scheme, β -ketonic acids, formed by oxidation of fatty acid on the β carbon, would give rise to methyl ketones (after decarboxylation), and these, by reduction of the carbonyl group, would lead to paraffins of one carbon atom less than the original acids. This scheme is therefore in accordance with the observation that paraffins

found in waxes from higher plants and insects have always been found to contain an odd number of carbon atoms. Although higher methyl ketones, corresponding to the paraffins from waxes have not been found so far, a number of such compounds of lower chain length have been isolated from plants, *e.g.*, methyl heptyl, methyl nonyl, methyl undecyl ketones. Since the above scheme was formulated, Pangborn and Anderson (94) isolated from acid-fast bacteria two secondary alcohols with an *even* number of carbon atoms, *i.e.*, *d*-2-octadecanol, $\text{CH}_3(\text{CH}_2)_{16}\text{CHOHCH}_3$, and *d*-2-eicosanol, $\text{CH}_3(\text{CH}_2)_{18}\text{CHOHCH}_3$. These alcohols could not be synthesized according to the above scheme unless one assumes that these bacteria, which are characterized by a very peculiar metabolism, are capable of forming straight-chain fatty acids with an odd number of carbon atoms. So far, whenever a claim has been made that *n*-fatty acids with an odd number of carbon atoms have been isolated, subsequent research showed them to be a mixture of near even-numbered homologs. Fatty acids, apparently belonging to the C_{19} , C_{21} , and C_{25} series (some of them optically active, suggesting branched chains), have been found in the fat of the leprosy bacillus (3) but very recent investigations of Stålberg-Stenhagen and Stenhagen (128), who studied the behavior of monolayers of fatty acids of acid-fast bacteria, suggest that only mixtures of normal-chain homologs are present. Further work on the metabolism of acid-fast bacteria may perhaps shed some light on the synthesis of such alcohols.

The synthesis of hydroxy and ketonic fatty acids found in both fats (*e.g.*, ricinoleic acid) and waxes (13-keto-*n*-dotriacontanoic acid) and also some secondary alcohols and ketones found in waxes could, according to Chibnall and Piper (24), take place from the corresponding unsaturated fatty acids. The following scheme, slightly modified to fit the more recent view that hydroxy fatty acids are not likely to be intermediates in the formation of keto acids from the corresponding fatty acids, expresses the essential features of the suggestions of these authors:

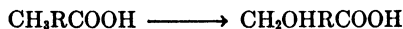


Since some of the hydroxy fatty acids found in nature have the hydroxyl group in the ω position (*e.g.*, sabinic acid, 16-hydroxyhexa-

decanoic acid, juniperic acid, 12-hydroxydodecanoic acid), they may arise in the course of ω oxidation (141) of the corresponding fatty acids, either indirectly, according to the view of Chibnall and Piper:



or by direct introduction of the hydroxyl group in the methyl group:



The latter mechanism would appear to be more likely in view of the work of Kuhn *et al.* (64,65), who found that in the course of the oxidation of the methyl group, in the animal, *e.g.* camphor the alcohol stage can be isolated and precedes the formation of the acid. Although so far ω oxidation of fatty acids in the animal body has been demonstrated only for some lower fatty acids (12), this mechanism, even for some higher fatty acids, might not be excluded in plants.

A word of caution should be added about the mechanism of the formation of hydroxy fatty acids. Until recently, the finding of β -hydroxy acids as metabolites of saturated or α,β -unsaturated fatty acids was taken as evidence that hydroxy fatty acids are intermediates in the oxidative degradation of fatty acids in the animal body. Thus, the isolation of β -phenyl- β -hydroxypropionic acid in animals fed β -phenylpropionic acid (26) gave support to the original conception of β oxidation of Knoop (62). The work of Jowett and Quastel (58), and later Blixenkrone-Møller (14) and Kleinzeller (59) suggested that β -hydroxybutyric acid is not an intermediate in the oxidation of butyric acid. The work of Friedmann (42) now showed that the steric configuration of the hydroxy compound has also to be taken into account. He found that in yeast acetoacetic acid is reduced to *d*- β -hydroxybutyric acid, although the natural β -hydroxybutyric acid belongs to the *l*-series. Some recent work of Friedmann (42a) has shown that β -phenyl- β -hydroxypropionic acid, isolated as a metabolite of β -phenylpropionic acid, also belongs to the *d*-series. Further thorough study of the steric configuration of hydroxy compounds found in nature (*e.g.*, methylalkylcarbinols, hydroxy fatty acids) may therefore be necessary to help elucidate the mechanism of their formation.

Waxes of higher plants and insects appear to represent mostly end products of metabolism, *i.e.*, they are secretions which do not seem to be appreciably further metabolized. The role of waxes of acid-fast bacteria does not seem quite clear, especially in view of the find-

ing that some of the constituents, e.g., phthioic acid (3,13,19-trimethyltricosanoic acid, 96a) produce in animals lesions characteristic for tuberculosis.

Even higher paraffins can, however, be utilized by some microorganisms. Thus Tausz and Peter (138) found that *Bacterium aliphaticum* quantitatively oxidizes *n*-hexane, *n*-octane, *n*-hexadecane, and *n*-triacontane, and also some higher unsaturated paraffins (octaene and hexadecene). More recently Tauson (137) found yeasts which can metabolize higher paraffins as their only carbon source. Stetten (132) found that also the animal organism can use paraffins, since he observed that, in rats fed deuteriohexadecane, deuterium was found in body fatty acids and other lipides.

The schemes suggested for the synthesis of the various components of plant and animal waxes would appear to be in accordance with the constitution of most of their individual constituents. Direct proof of the convertibility of fatty acids in higher alcohols, ketones, paraffins, etc., according to the above or possibly other schemes still await further research in this very difficult field.

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THE BIOCHEMISTRY OF FATTY ACID CATABOLISM*

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I. Introduction

Among the naturally occurring normal fatty acids, saturated and unsaturated straight-chain fatty acids of sixteen and eighteen carbon atoms occur most frequently; those with twelve to fourteen and twenty to twenty-four carbon atoms are less frequently encountered. The almost complete absence in naturally occurring fats of fatty acids having five to twelve carbon atoms is particularly striking. A list of quantitative data will be found in the paper by Smedley-Maclean

* In memory of Franz Knoop, 1875-1946.
Translated from the German.

(251). Except for a few acids of low carbon content (C_5 , C_7 , C_9), fatty acids occurring in nature do not have an odd number of carbon atoms.

It can be assumed that fatty acid catabolism occurs in the tissues by way of chemically defined reactive intermediates. The concentration of these intermediary compounds must be inversely proportional to the activity of the enzymes that act on the substance. It is therefore to be expected that the most important intermediate compounds concerned with intermediary metabolism in the living tissue occur only in small concentrations. This is the reason why some of them may have eluded isolation up to now. Since enzyme equilibria are not always alike, intermediate products of fatty acid catabolism can be expected to accumulate in some types of cells. This may also be true of products of a side reaction not involved in the principal metabolic process. As a result, it is possible to obtain an indication of possible intermediate products involved in fatty acid catabolism from the constitution of naturally occurring substances similar to fatty acids.

It will therefore be useful to prepare first a list of the known fatty acid-like substances occurring in nature. The following such groups are known in addition to the normal fatty acid phosphatides:

(1) Fat aldehydes in the form of plasmalogens bound to glycerophosphate as acetals (97). Also α,β -unsaturated fatty aldehydes, like the α,β -hexylene aldehyde found in plant leaves (66, 221; equation 34, page 369). (2) Lipide alcohols occurring in waxes, such as cetyl alcohol and others, as well as the α,β -unsaturated hexylene alcohol found in the oil of tea leaves (240). (3) Methyl ketones, which almost always have an odd carbon number. These probably are a result of the decarboxylation of even-numbered β -keto fatty acids (290). Aromatic substances in many plant oils and the paraffins obtained from plant waxes which also generally have an odd number of carbon atoms and which, according to Chibnall and Piper (60) may have had their origin in the higher ketones. In addition there are many keto fatty acids and ketone alcohols found in plant waxes which have a fairly high molecular weight (28,60). (4) α -Hydroxy fatty acids derived from phrenosin and kerasin (61,150) as well as β -hydroxydecanoic acid which Bergström, Theorell, and Davide (11) isolated from *Pseudomonas pyocyanea* (equation 13, page 357); 9,10-dihydroxystearic acid (147) and aleuritic acid (214,234, 294); equation 36, page 372). (5) Fatty dicarboxylic acids, C_9 to

C₁₁, such as were obtained by Verkade and co-workers (297-300) from the urine of animals fed simple fatty acids of medium chain length. It is conceivable that these acids also occur in normal metabolism in small quantities (eq. 38, page 375). In addition, there occurs an unsaturated α,β -dodecylenedicarboxylic acid, traumatic acid, which English, Bonner, and Haagen-Smit (88,89) isolated as a wound hormone in plants (equation 33, page 368). (6) Simple or multiple unsaturated fatty acids, with the first double bond after the carboxyl group usually in the 9,10 position: oleic acid, linoleic acid, linolenic acid, ricinoleic acid, and others (equation 35, page 370); arachidonic acid, a 5,6,8,9,11,12,14,15-tetraenoic C₂₀ acid (eq. 50, page 385), and aleuritic acid, a 9,10- ω -trihydroxy fatty acid, also belong in this group. (7) Fatty acids with branched chains occurring in the tuberculin lipides (2) and also branched fatty alcohols, aldehydes and ketones, such as geraniol, citral, etc., as well as the squalenes occurring in the livers of certain fish. This group also includes 2-ethyl-2-dodecylethanol and 2-hexyl-2-octylethanol found in the liver of the deep sea fish *Ruwettus pretiosus* (192). (8) Agaricinic acid, a paraffin-substituted citric acid occurring in *Polyporus officinalis* (Fries) (238,291; equation 58, page 394). Spiculosporic acid isolated by Clutterbuck, Raistrick, Rintoul (64); minioluteic acid (21, equation 58), homologs of citric acid which have paraffin chain substitutes, and, finally, γ -ketopentadecanoic acid.

Investigating the biochemistry of the fatty acid catabolism in the animal is more difficult than investigating carbohydrate or protein metabolism and therefore has lagged behind. This has been caused by several factors: (1) Fatty acids and their salts are difficultly soluble in water; this fact, together with the slow diffusibility of fatty acids through the cell walls, has made it difficult experimentally to bring the substrate and the enzymes together. Therefore incubation of macerated tissue or tissue sections with fatty acids has produced few results. (2) The poisonous action on tissue respiration of saponified and therefore water-soluble fatty acids permits only low substrate concentrations in *in vitro* incubation experiments and may bring about reactions which are of no consequence *in vivo*. (3) The quantitatively relatively small conversion capacity of the tissue enzymes with respect to fatty acids, as compared to their capacity to act on carbohydrates and proteins. (4) The lack of suitable microanalytical methods for the proper differentiation, isolation, and

identification of small quantities of metabolized fatty acids and of their metabolites.

As a result of these difficulties most investigations on the biochemistry of the fatty acids so far have not been single incubation experiments with individual tissues, but have been feeding experiments with the whole animal. This was the method used in the discovery of the classical β oxidation and ω oxidation. Unfortunately, experiments on the metabolism of the whole animal yield only limited information on the mechanism of intermediary metabolism, since several organs with varying chemical action take part in the metabolism in an unpredictable manner and sequence. Add to that the fact that 60–100% of all substances fed in these experiments disappeared during intermediary metabolism. It is just these products which have disappeared, and their derivatives, which can give us most information about the fatty acid catabolism and the mechanism of fatty acid synthesis that may be connected with it.

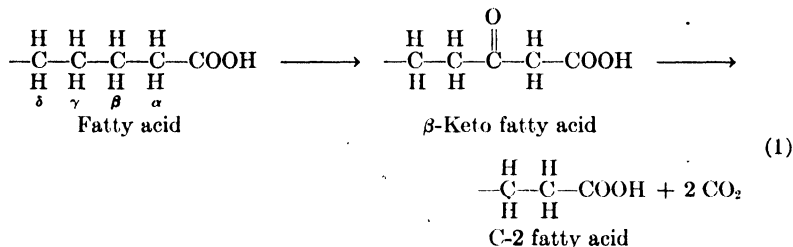
Further development of the biochemistry of fatty acids will therefore continue to depend on experiments with short incubations of individual tissues with fatty acids and subsequent painstaking microisolation of the products of reaction, even though these methods have so far been relatively unproductive. Then, after the mechanism has been understood, the enzymes involved will have to be isolated and studied one by one, as has already happened to a large extent in the biochemistry of sugar catabolism. Microanalyses based on colorimetry, respirometry, the measurement of respiratory quotients and the like can be valuable aids in this research. They have little value, however, unless the chemical substance has been isolated beyond doubt and the reaction products have been identified. In this connection we may call attention to a report of Bach (7d), to which little attention has been paid so far. He showed how questionable calculated respiratory quotients can be.

This paper will discuss only the biochemistry of the catabolism of the fatty acids, making the implied and probable, but not yet proved assumption that only the free fatty acids, and not the triglycerides, are catabolized. It is not yet clear whether phosphorylated intermediaries are involved, such as phosphatides or lipide aldehyde acetalphosphatides, *i.e.*, the plasmalogens or acyl phosphates. For further reference, in addition to the yearly reports on the biochemistry of fats contained in *Annual Review of Biochemistry*, special atten-

tion is called to the concise book by Smedley-Maclean (252), *The Metabolism of Fat*, and the detailed treatise of Bloor (27), *Biochemistry of the Fatty Acids*, which have appeared recently.

II. β Oxidation

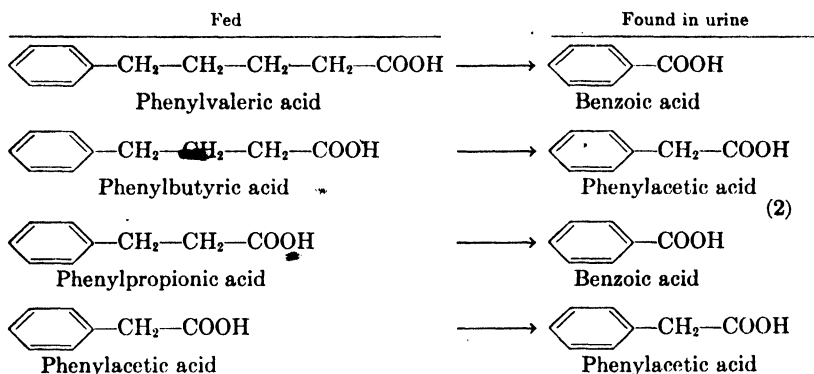
The first step toward an understanding of the metabolism of fatty acids was made by Franz Knoop in 1906 (152) in his classic paper on β oxidation. He found that, contrary to all previous assumptions concerning the mechanism of the oxidation of the fatty acid molecule in the body, the carbon atom which had the β position with respect to the carboxyl group was most readily attacked by the cells.



In spite of its experimental verification the β oxidation theory did not at first meet with ready acceptance. It was accepted only when, in 1909, Dakin (67) succeeded in showing that the protons located at the β -carbon atom can be most easily replaced by oxygen, even when the fatty acid was merely subjected to a purely chemical treatment with hydrogen peroxide. This shows that in this case, too, the protons at the β -carbon atom are more readily replaced than those at the α -carbon (just the opposite of what takes place when bromine is substituted). Later investigations have on the whole confirmed these purely chemical findings of Dakin. Ponsford and Smedley-Maclean (226) found that β -keto acids were formed when they oxidized suberic acid (obtained from cork, a C_8 -dicarboxylic acid) with hydrogen peroxide at $60^\circ C$. Wieland (310) found the same when oxidizing α, β -unsaturated fatty acids. Clutterbuck and Raper (63), on the other hand, were able to identify γ - and δ -keto acids as well as β -keto acids when they oxidized palmitic acid with hydrogen peroxide.

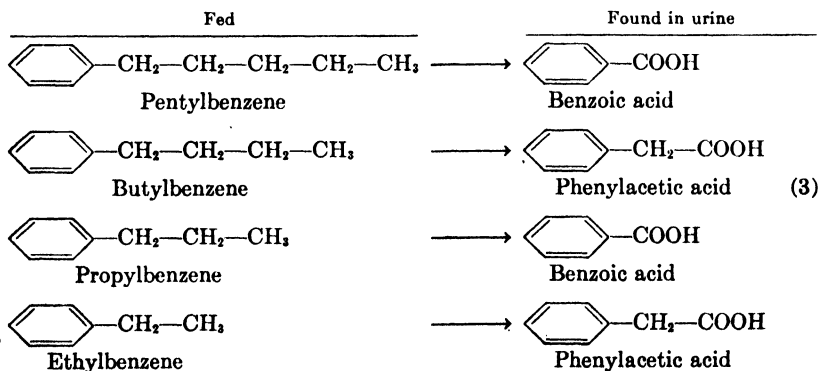
Knoop based his theory on results obtained from feeding phenyl derivatives of fatty acids to dogs, after isolating and identifying the metabolites excreted in the urine. He found (see eq. 2) that, when

he fed ω -phenylvaleric acid, he obtained benzoic acid, while ω -phenylbutanoic acid gave phenylacetic acid; ω -phenylpropionic acid gave benzoic acid and phenylacetic acid was excreted unchanged as phenylacetic acid. These products also occurred in part in the form of glycine derivatives, *i.e.*, as hippuric acid and phenaceturic acid.



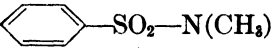
In each case, as many two-carbon fragments were oxidized off as the nonreactive benzene ring permitted. Raper and Wayne (233) confirmed these experiments in 1928 and extended them to cover higher homologs. When these were used, yields of hippuric acid and phenaceturic acid were lower, a large portion of the acids which were fed to the animals having been completely oxidized in an unknown fashion.

Thierfelder and Klenk (286) extended these findings further. When alkyl benzenes are fed, the methyl end groups apparently undergo a primary oxidation to a carboxyl group and then undergo



β oxidation to become benzoic and phenylacetic acid derivatives. The authors found the results shown by reactions (3).

Dicarboxylic acids also underwent β oxidation. When sebacic acid (C_{10} -dicarboxylic acid) was fed to dogs, Flaschenträger (102) found suberic acid (C_8 -dicarboxylic acid) and adipic acid (C_6 -dicarboxylic acid) in the urine, in addition to 50% of the acid which was excreted unchanged. This then meant that a two-way β oxidation had taken place.

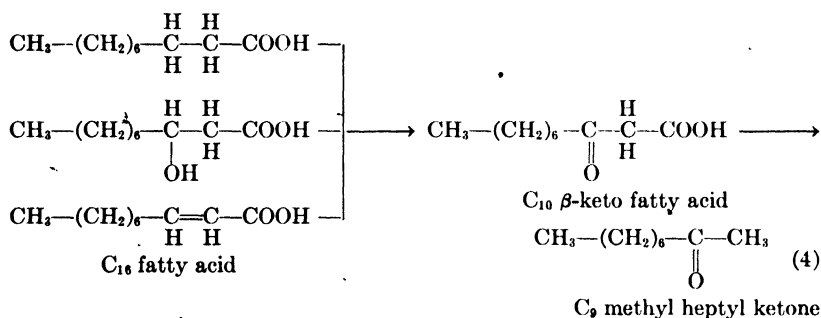
Thomas and Schotte (289), in 1919, were the first to find that, when substituted ω -amino acids were fed, repeated β oxidation could occur to an extent that even-numbered fatty acids gave ω -aminobutanoic acid, while acids with an uneven number of carbon atoms gave ω -aminopropanoic acid. Students of Thomas, *e.g.*, Flaschenträger and co-workers (105), extended these experiments in order to cover nitrogen-substituted ω -amino acids, such as  $-(CH_2)_x-COOH$. They obtained the same results; depending on whether the fatty acid contained an even or an odd number of carbon atoms, either the substituted butanoic or propanoic acid $-(CH_2)_x = (CH_2)_3$ or $(CH_2)_2$, respectively—was isolated from the urine. In every case in which anything was excreted at all, β oxidation took place. So far no feeding experiment succeeded in establishing what happens to the completely oxidized part of the fatty acid. Levey and Lewis (184) report that when ω -phenoxyacaproic acid was fed to rabbits, free β phenoxyacetic acid was excreted.

Bernhard and Vischer (20) found that when they fed behenic acid-*d* (C_{22}), stearic acid-*d* (C_{18}) was found in the depot fat. Bloch and Rittenberg (25,26) noted that only even-numbered fatty acids containing deuterium resulted in acetic acid-*d* in the tissue after catabolism, but that this was not the case with fatty acids containing an odd number of carbon atoms. See Section XI for a possible explanation of this phenomenon.

According to Wakeman and Dakin (303), liver extracts oxidize β -hydroxybutanoic acid. The reverse reaction, *i.e.*, the reduction of acetoacetic acid to β -hydroxybutanoic acid, also takes place in the liver (68). Muñoz and Leloir (212) also discuss liver preparations which oxidize butanoic acid. Stable preparations obtained from pig hearts which oxidize β -hydroxybutanoic acid were prepared by Hoff-Jørgensen (127). Active extracts have also been obtained from rat kidneys (213). Lang (171) was able to show that such preparations

not only dehydrate β -hydroxybutanoic acid, but also any β -hydroxy fatty acid containing up to nine carbon atoms. Breusch and Tulus (44) found in Thunberg experiments that cat liver also dehydrates β -hydroxy fatty acids containing up to fourteen carbon atoms. Breusch and Tulus were able to report later (46), that when macerated muscle and liver tissues were subjected to short incubations, α - and β -keto fatty acids disappeared, while this was not the case with γ - and δ -keto fatty acids. It remains to be investigated how keto acids not catabolized in a short experiment would behave under conditions of long-term experiments, such as feeding experiments. Phenylated γ -ketoheptanoic acid, when fed, underwent a normal β oxidation, unaffected by the presence of the γ -keto group (155). In addition, hydrogenations and keto group reductions take place.

Thoms (290) was the first to isolate higher methyl ketones from leaf oils; later Haller and Lassieur (119) did the same with coconut fat; subsequently Stärkle (263) was able to show that these ketones are formed by the action of *Penicillium glaucum* on fatty acids which are oxidized to β -keto fatty acids and then split off carbon dioxide (see also Acklin, 1).

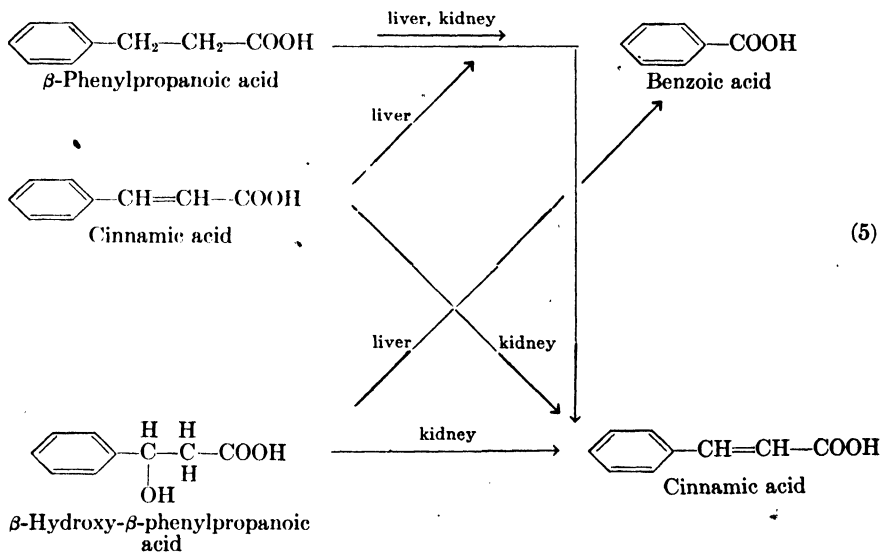


Later Thaler and Geist (283) were able to show that β -hydroxy fatty acids, such as pure β -hydroxycaproic acid or α,β -unsaturated caproic acid, C_{10} (282), are also decarboxylated in part to higher methyl ketones by *Penicillium glaucum*.

It has not yet been determined whether the first products of this β oxidation are β -hydroxy fatty acids or β -keto acids directly; the product may also depend on the organ in which the reaction is taking place. According to Snapper, Grünbaum, and Neuberg (256), when butanoic acid is oxidized to acetoacetic acid in the kidneys, no hy-

droxybutanoic acid is formed as an intermediate. Quastel and Jowett (139) obtained the same results with liver sections; this was confirmed by Leloir and Muñoz (182) on liver preparations. Quastel and Wheatley (232) report that only intact liver sections are capable of oxidizing fatty acids, while macerated liver section cannot do so; this of course makes experimentation more difficult.

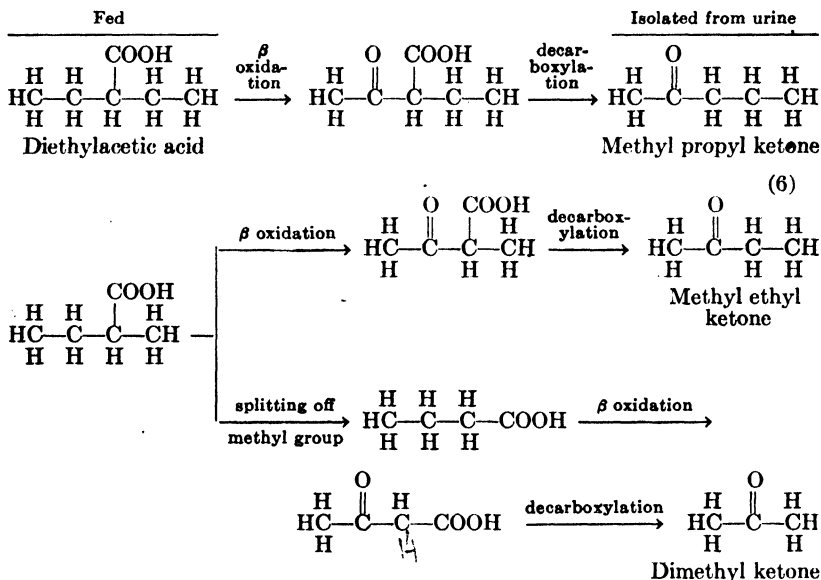
In diffusion experiments on liver and kidney sections, Snapper and Grünbaum (258) found that phenylpropanoic acid and β -phenyl- β -hydroxypropanoic acid, as well as cinnamic acid, are oxidized to benzoic acid. Only phenylpropanoic acid and cinnamic acid gave benzoic acid in the kidneys, while β -hydroxy- β -phenylpropionic acid splits off water and is primarily excreted in the form of the glycine derivative of cinnamic acid (eq. 5).



According to Kuhn and Livada (167), α -methylated ω -phenyl fatty acids undergo β oxidation, partially splitting off the methyl group. β -Methylated fatty acids, on the other hand, are difficult to oxidize and are mostly excreted unchanged in the urine, though they do undergo ω oxidation in part. It is not known what happens to the portion which is missing and does not reappear in the urine. Lang and Adikes (174), however, incubated tissue sections with α -methyl fatty acids without phenyl and obtained only very few ketone bod-

ies, but obtained many when the tissue sections were incubated with β , γ , or δ -methyl fatty acids. In view of the complex mechanism of ketone formation, a mere demonstration of ketone bodies, without identification of the ketones and a knowledge of the mechanism of their formation, unfortunately does not tell us much concerning fatty acid catabolism (see Section XI). On the subject of methylations, see Bach (7e).

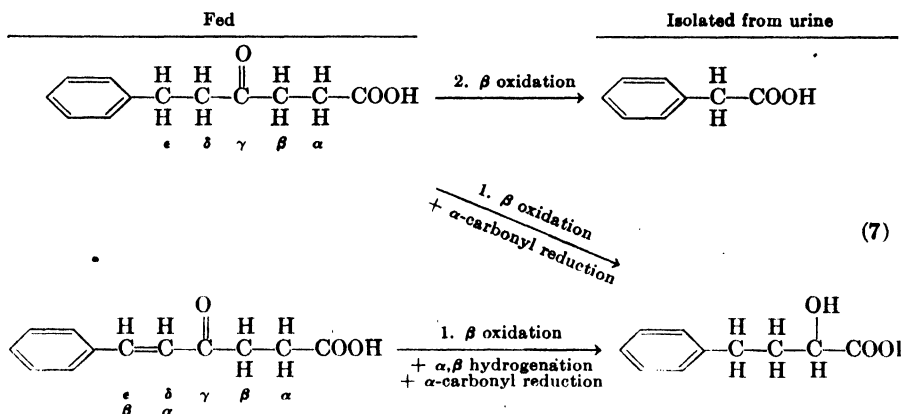
Experiments of Blum and Koppel (30) have shown that the animal organism is readily capable of splitting off methyl side chains, but unable to do so with ethyl side chains. When the authors fed dogs diethylacetic acid, they were able to isolate methyl propyl ketone in the urine (identified as *p*-nitrophenylhydrazone), but when they fed methylethylacetic acid, only methyl ketone was found (eq. 6).



This result can only be explained if we assume that β oxidation takes place in the first instance, without regard for the ethyl group in the α position, while in the second instance, the methyl group is split off before β oxidation takes place. If this were not the case, methyl ethyl ketone—not dimethyl ketone would have been formed.

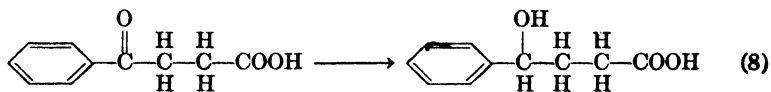
In addition to β oxidation, there also take place in the body (eq. 7) reductions of the double bonds which are in α, β position to the car-

bonyl groups, and there also occur reductions of the carbonyl groups (155).



Thus, both γ -keto- ω -phenylhexanoic acid and γ -keto- Δ - δ - ϵ , ϵ -phenylhexanoic acid yield phenylacetic acid and α -hydroxy- γ -phenylbutanoic acid after passing through the body, but form no trace of benzoic acid. The γ -keto group has not interfered with the normal course of the β oxidation. After undergoing β oxidation and thus splitting out two carbon atoms, the γ -keto group becomes an α -keto group and can readily undergo reduction in this form.

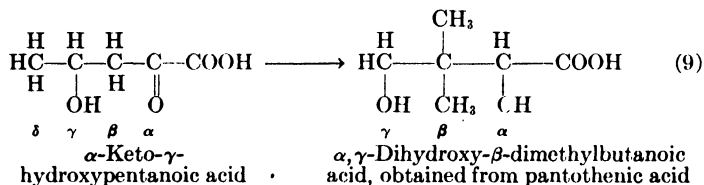
The only known biochemical reaction of a γ -keto group (eq. 8) is the reduction after feeding of γ -keto- γ -phenylbutanoic acid (benzoylpropanoic acid) to γ -hydroxy- γ -phenylbutanoic acid, reported by Thierfelder and Schempp (285). In this case, however, the proximity of the benzene ring has resulted in a change of the electrical conditions. Thus, after feeding, acetophenone is oxidized to benzoic acid in part (eq. 62, page 402), while no such oxidation has been reported for any other methyl ketones.



Witzemann (314) has advanced a theory of fatty acid catabolism according to which α , γ -diketo fatty acids are formed first from the fatty acids and then oxidized by way of α , β , γ -triketo fatty acids. According to Krebs and Johnson (160), α , γ -diketopentanoic acid can form acetoacetic acid and β -hydroxybutanoic acid, after splitting

off carbon dioxide, by the action of enzymes. This has been confirmed by Lehninger (178). Lehninger also found that the animal organism burns largely α,γ -diketopentanoic acid. In cases of insulin hypoglycemia, this acid, just like glucose, helps save the life of the patient. Investigations by Breusch and Ulusoy (49) have shown that α,γ -diketohexanoic acid and macerated liver tissue do not form acetone, even in traces, while β,δ -diketohexanoic acid forms acetoacetic acid and acetone in almost quantitative proportions (see Section III). Since, according to Dakin (69), hexanoic acid, β -hydroxyhexanoic acid and α,β -unsaturated hexenoic acid form acetoacetic acid with the aid of liver tissue, it seems improbable that the reaction in the liver takes place in the way of an α,γ oxidation, at least as far as hexanoic acid is concerned. The higher α,γ -diketo fatty acids—up to C_{16} —which Breusch and Keskin synthesized (42), undergo primarily a reduction of the α -keto groups in muscle and liver tissues (Breusch, unpublished data).

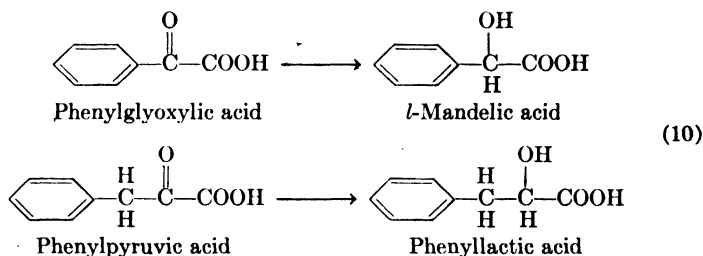
Some acids of animal origin are known, where the possibility of an α,γ -oxidation can still be postulated, at least as an alternative side path for the oxidation of special fatty acids. The acids referred to are α -keto- γ -hydroxypentanoic acid (eq. 9), one of the products of glucose metabolism of *Staphylococcus albus* which was isolated by Fosdick and Rapp (107), and α,γ -dihydroxy- β -dimethylbutanoic acid which occurs as a natural constituent of pantothenic acid (268,312); there also occurs licanic acid, a γ -ketolinolenic acid (311a).



So far it has not been possible to demonstrate clearly α oxidation of fatty acids, with the exception of the enzymic oxidation of propanoic to lactic acid (29; see however 153). Nevertheless, we must count on the possibility in principle that oxygen can also enter the fatty acid molecule at the α position, though in a manner as yet unknown to us, as is indicated by the existence of hydroxynervonic acid—an unsaturated α -hydroxytetracosanoic acid, with the double bond in 9,10 position, found in the cerebroside, phrenosin (61,150). The existence of α -amino fatty acids may also be an indication in this direction.

Pig kidneys, on the other hand, contain an L - α -hydroxy acid oxidase, a flavoprotein, which possibly is identical with an α -amino acid oxidase (22,62,140,242). This enzyme acts only on α -hydroxy acids, but not free fatty acids and thus is not involved in the catabolism of fatty acids. The reaction products are α -keto acids. Any further oxidation which would lead to fatty acids possessing an odd number of carbon atoms has not been reported, except for the low-carbon α -keto fatty acids (112,113). Except for phenyllactic acid, only α -hydroxy acids between C_4 and C_6 were investigated as substrates. The enzyme is not identical with lactic acid dehydrogenase.

The reverse reaction also takes place, *i.e.*, the reduction of α - and β -keto groups. Neubauer (215) reports that when phenylglyoxylic acid was fed it was reduced to L -mandelic acid and excreted in this form. Phenylpyruvic acid is reduced (eq. 10) to phenyllactic acid (278).

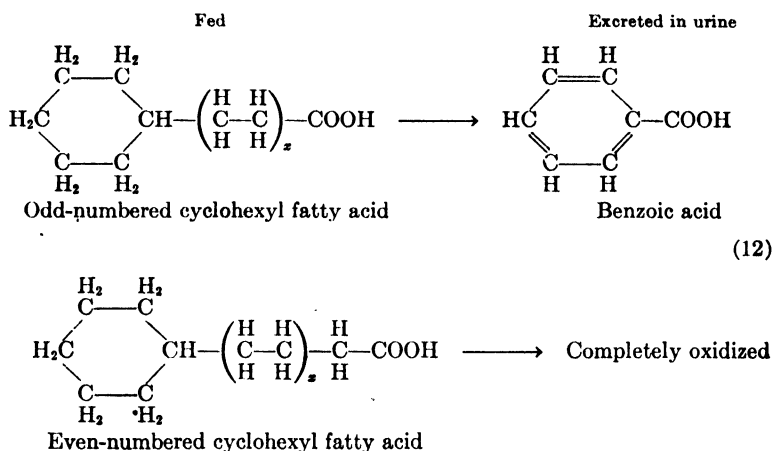


Generally speaking, α - and β -keto acids are readily reduced to hydroxy acids in liver, muscle, and kidney, but not so in lung, spleen, and placenta (46); the same applies to oxalacetic acid, α -ketoglutaric acid, and oxalocitramalic acid (eq. 59, page 395), in addition to pyruvic acid. When ingested, β -keto- β -furanpropionic acid is reduced to the corresponding β -hydroxy- β -furanpropionic acid and is excreted in this form in the urine (243).

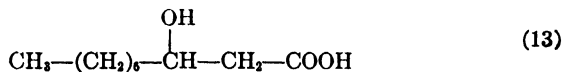
Acetoacetic acid can be reversibly reduced to β -hydroxybutanoic acid by liver and muscle tissues, as was first reported by Neubauer (216).

Few reductions are known which go so far as to reduce hydroxyl and keto groups to CH_2 groups. *Clostridium acetobutylicum* reduces acetoacetic acid to butyl alcohol (317). It is possible that such reductions are involved in the biosynthesis of fatty acids. Chibnall and Piper (60) explain the occurrence in plant waxes of paraffins which

undergoing β oxidation. Cyclohexyl fatty acids with an even number of carbon atoms are completely oxidized, like tyrosine. It is worth while pointing out the similarity of these acids to chaulmoogra acid.



The higher methyl ketones, like methyl heptyl ketone, methyl nonyl ketone, etc. (equation 4, page 350), which occur in nature in many plants as aromatic substances, are proof of the existence of β oxidation. They originated as a result of the decarboxylation of high β -keto fatty acids and have generally an uneven number of carbon atoms, as they come from fatty acids with an even number of carbon atoms. Another substance in this group is (–) β -hydroxydecanoic acid (formula 13) which Bergström, Theorell, and Davide (11) recently isolated from *Pseudomonas pyocyanea*.

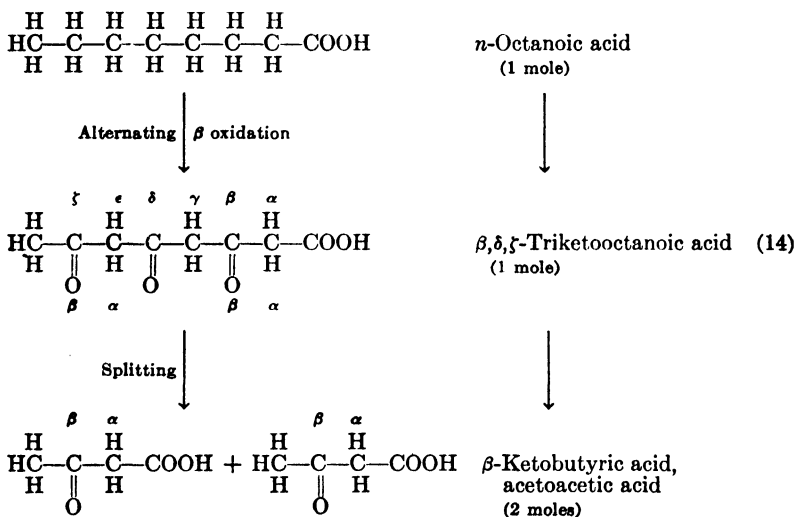


On the basis of these experiments it can therefore be taken as proved that enzyme systems exist in both plant and animal cells which are capable of nonspecifically oxidizing the β -CH₂ group of fatty acids into a β -CHOH group, a β -C=O group, or a COOH group. β Oxidation takes place primarily in muscle tissue, kidneys, and liver. Nothing is known about the actual chemical mechanism; so far, none of the enzymes involved has been isolated, nor have their reactivities been determined.

III. Alternating β Oxidation

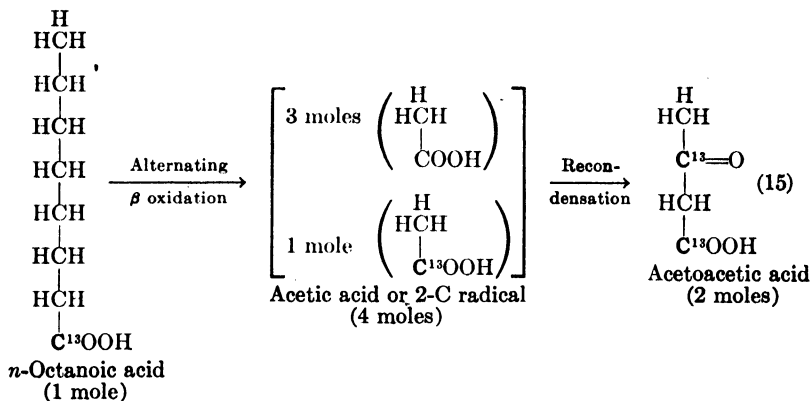
The theory of simple β oxidation of fatty acids did not prove adequate to explain the great capacity of the liver to form ketone bodies, especially acetoacetic acid, from fatty acids.

In 1916, Hurtley (136) had already postulated that, in addition to oxidation of the β carbon of the fatty acid chain, in animal catabolism oxidation also occurs at every other second C atom following β carbon. This hypothesis of alternating β oxidation was again forgotten, since no further experimental evidence was forthcoming and only in 1935 received new experimental proof in the hands of Jowett and Quastel (138,139). They found that *n*-octanoic acid (caprylic acid) yielded more than one mole of acetoacetic acid when acted upon by liver, although it should have yielded at most one mole, according to the classical theory. They therefore postulated that the fatty acid chain yields as many moles of acetoacetic acid after undergoing alternating β oxidation as its carbon number can be divided by four. Butts, Cutler, Hallman, and Deuel (54) suggested in the same year that δ as well as β oxidation takes place. Blixenkrone-Møller (24) and Lehninger (180) confirmed the experimental findings of Jowett and Quastel. See reaction (14).



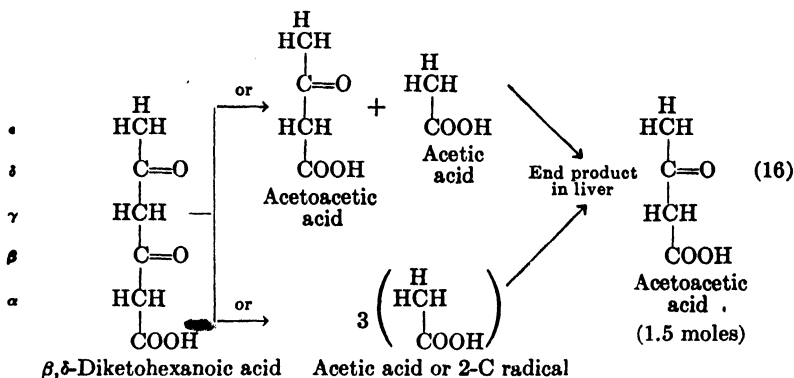
Weinhouse, Medes, and Floyd (204,306,307) repeated these experiments, using an octanoic acid the carboxyl groups of which contained

the heavy carbon isotope C^{13} (see eq. 15); they found that the acetoacetic acid which they isolated contained the isotope not only in the carboxyl group, but also in the β -carbonyl group. The carboxyl group was found to contain slightly more C^{13} than the carbonyl group. This then proved that, in the event of alternating β oxidation in the liver, acetoacetic acid is not formed directly for the most part, nor are four-carbon residues formed, as had been assumed by Jowett



and Quastel, but rather that first an oxidative splitting into two-carbon fragments takes place, and subsequently recondensation to acetoacetic acid occurs. It has been proved repeatedly that acetoacetic acid can be formed from acetic acid or two-carbon fragment radicals (see Sections X and XI).

It is impossible to test the second part of the theory by incubating liver sections with β,δ,ζ -triketooctanoic acid, since this readily decomposable acid is not yet available synthetically. The corresponding β,δ -diketohexanoic acid (triacetic acid), on the other hand, is well known. Breusch and Ulusoy (49) were able to show that this acid is rapidly and quantitatively metabolized to acetoacetic acid by macerated liver tissue (300–600 mg./100 g. cat liver/hour \approx 300 g. for an adult man per day). Kidney, muscle, and brain tissue have practically no effect on the acid. Hence it is only the liver and no other tissue which readily decomposes triacetic acid to three two-carbon fragments in such a way that both keto groups are transformed to carboxyl groups, after the chain has split (reaction 16). Thus there are two possibilities: either three moles of acetic acid or three two-carbon radicals are formed, with subsequent recondensation to aceto-



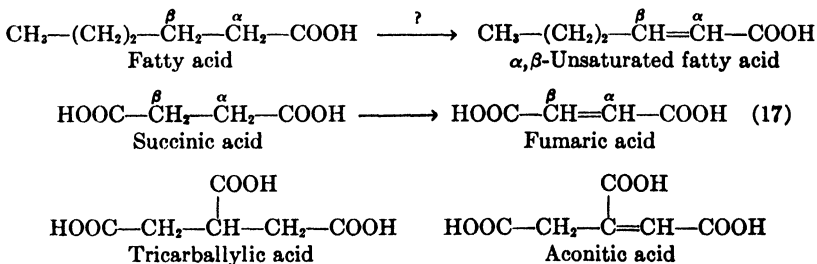
acetic acid, or one mole of acetoacetic acid is formed directly, while one mole of acetic acid is formed, which then is recondensed to acetoacetic acid. The latter possibility is less probable, however.

One therefore can conclude that the catabolism of fatty acids in the liver differs basically from that in either the muscles or the kidneys, similar to what Breusch found in the case of citric acid formation (38,39; see Section X).

Thus, alternating β oxidation of the fatty acid chain is probably the primary degradation mechanism in the liver. This is not the case in fatty acid catabolism in muscle and kidney tissue.

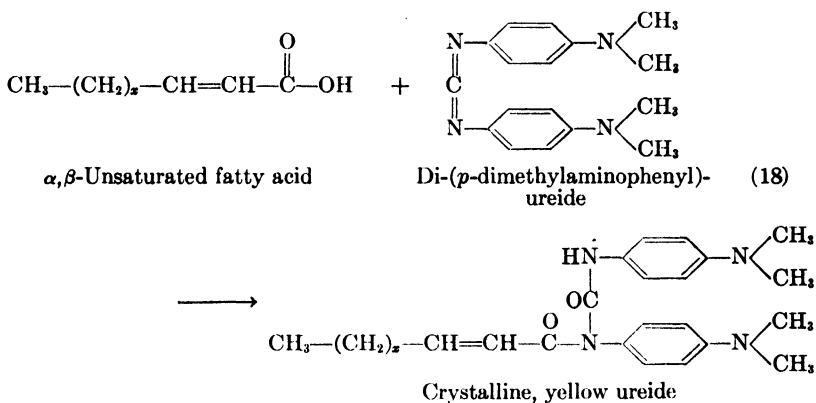
IV. α, β Dehydrogenation

The suggestion has frequently been advanced that α, β dehydrogenation accompanies or precedes the β oxidation of fatty acids (*e.g.*, 229,230); this is supposed to be analogous to the biochemical α, β dehydrogenation of succinic acid to fumaric acid (reactions 17). The analogy is questionable since tricarballic acid, a tricarboxylic acid homologous to succinic acid, is not dehydrogenated by muscle

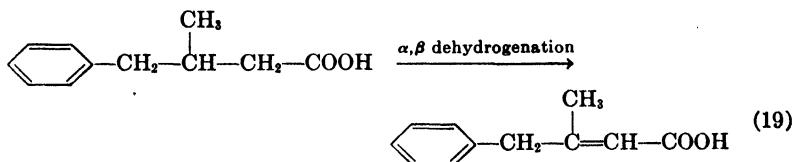


tissue to the corresponding unsaturated acid, namely, aconitic acid (37). Quastel (231) was able to show slight dehydrogenation of tri-carballylic acid in Thunberg experiments on *Escherichia coli*.

So far no enzyme system has been discovered which specifically dehydrogenates the α,β position in fatty acids. This may be due to difficulties of analytical methods, since steric interference in ordinary α,β -unsaturated fatty acids causes the ordinary methods of proof for the presence of fatty acid double bonds, such as halogen addition, to fail. This was first shown by Ponzio and Gastaldi (227) and then confirmed by Tulus (295). Zetzsche and associates (319) have presented a method whereby α,β -unsaturated fatty acids can be characterized by their precipitating as yellow di-(*p*-dimethylaminophenyl)-ureides (eq. 18), while saturated fatty acids give colorless ureides. Breusch and Ulusoy (48) worked out the method in detail and introduced it to the chemistry of fats. It permits precipitating even oily fatty acids as crystalline ureides directly from an ether solution.

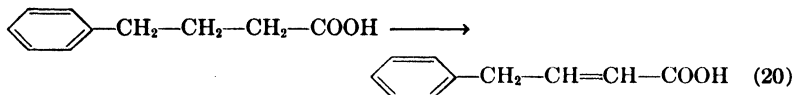


A paper of Carter, Osman, Levine, and Gamm (55) can be cited as evidence of the capacity of the animal to effect α,β dehydrogenation. The authors found (eq. 19) that β -methylated phenyl fatty acids are not oxidized but are excreted in part in the form of α,β -unsaturated

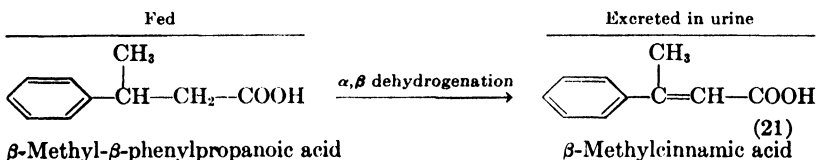


acids. This report is only a preliminary one, containing no isolation data, at least so far as the literature is available to this author.

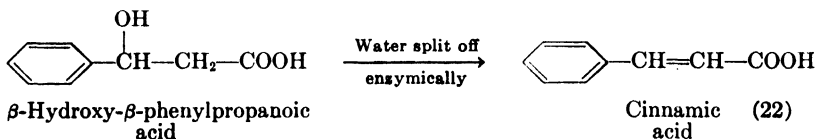
Mazza (202) succeeded in directly isolating phenylcrotonic acid in an experiment in which he incubated phenylbutanoic acid with liver extracts (reaction 20).



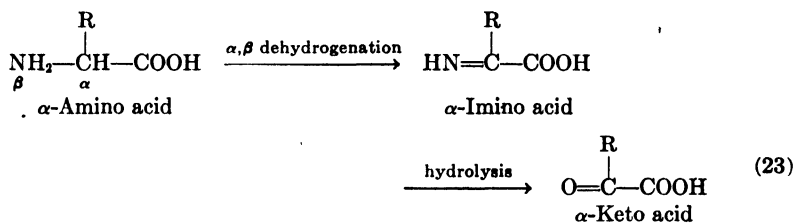
Kuhn and Livada (167) also observed α,β dehydrogenation. They were able to isolate 3% β -methylcinnamic acid, in addition to 50% original acid, after having fed dogs β -methyl- β -phenylpropanoic acid (eq. 21). It is not yet certain whether the α,β protons were



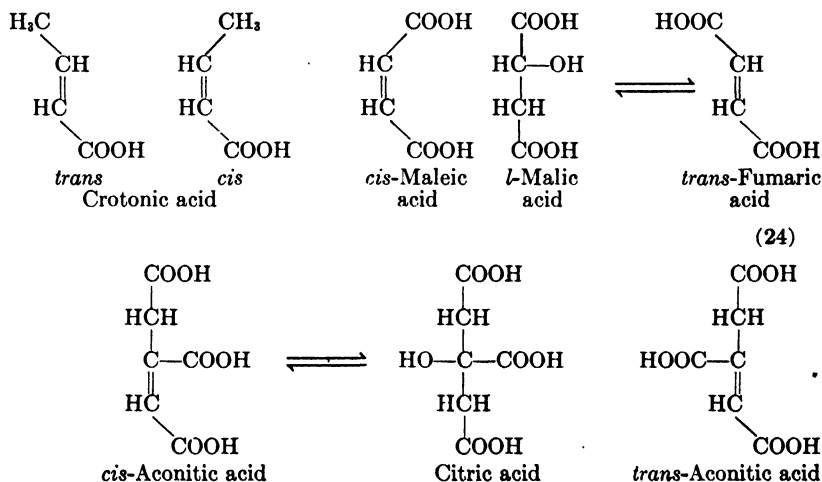
oxidized directly, or whether the acid first underwent α,β oxidation to the β -hydroxy acid and the α,β double bond originated by subsequent splitting off of a water molecule in the body or perhaps even in the preparation of the urine extracts in the laboratory. A paper of Snapper and Grünbaum (259) supports the thesis that the water is split off in the body (eq. 22). They found that when they diffuse kidneys with β -phenyl- β -hydroxypropanoic acid, water was split off and cinnamic acid was formed.



A similar enzymic dehydration has not as yet been observed in nonphenylated hydroxy fatty acids, but this has not been investigated. One may also consider the dehydrogenation of α -amino acids, although proceeding by means of other enzymes, as an α,β dehydrogenation (reaction 23).



The reverse reaction, *i.e.*, the addition of water to α,β double bonds with the formation of β -hydroxy fatty acids, is improbable, according to Lipman and Perlman (188), since α,β -unsaturated crotonic acid is not converted to β -hydroxybutanoic acid in tissue incubation experiments and is not in equilibrium with it. If there is no enzyme system for the lowest homologous members of a series, it is improbable that there is one for the higher homologs. This does not definitely solve the question, since Lipmann and Perlman studied only one crotonic acid, and not both *cis*- and *trans*-crotonic acid, though all enzyme systems concerned in these reactions have been found so far to exhibit very definite *cis-trans* specificity. Thus, the enzyme fumarase can only transform the *trans* acid, *i.e.*, fumaric acid, to *l*-malic acid, while it will not act on the *cis* acid, *i.e.*, maleic acid (eq. 24). Aconitase, on the other hand, acts only on *cis*-aconitic acid,

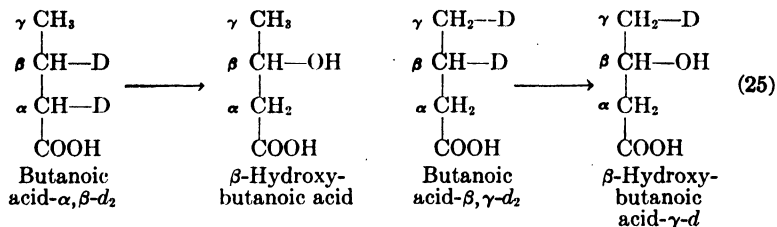


which is stable for short periods only, transforming it by the addition of water mostly into an equilibrium mixture of citric acid and isocitric

acid, while it will not act on the more common *trans*-aconitic acid, which is stable in solution.

In the liver, ordinary crotonic acid is oxidized directly to β -ketobutanoic acid, *i.e.*, acetoacetic acid, without the intermediate formation of β -hydroxybutanoic acid. According to Lipmann and Perlman, the reverse reaction, *i.e.*, the reduction of crotonic acid to butanoic acid, probably takes place in the kidneys.

Experiments of Morehouse (210) also tend to support the assumption of α,β desaturation. Morehouse fed butanoic acids containing deuterium in α and β positions. When he analyzed the β -hydroxybutanoic acid isolated from the urine he found it to contain only 4% of the deuterium. On the other hand, when he fed butanoic acids containing deuterium in β and γ positions, under similar conditions, he obtained a β -hydroxybutanoic acid, which contained five times more deuterium than before. It is possible, however, that when butanoic acid is oxidized β -ketobutanoic acid is formed directly. The enol form of this compound may readily exchange its deuterium in the α position with protons of ordinary water, β -hydroxybutanoic acid being formed by reduction only as a second step (see reaction 25).

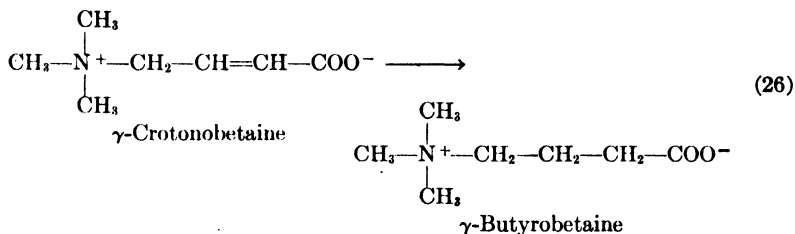


For this reason these experiments are not a definite proof of the occurrence of α,β dehydrogenation. They do prove, however, that the γ position is attacked much less readily, if at all, than the α and β positions, as would be expected on the basis of the theory of β oxidation.

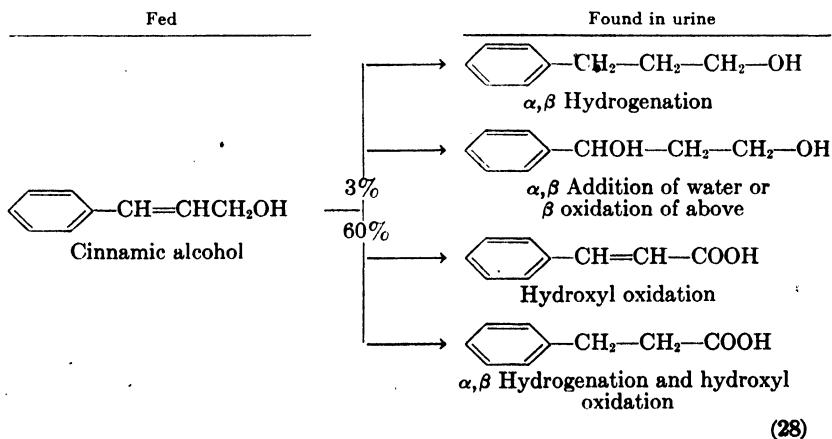
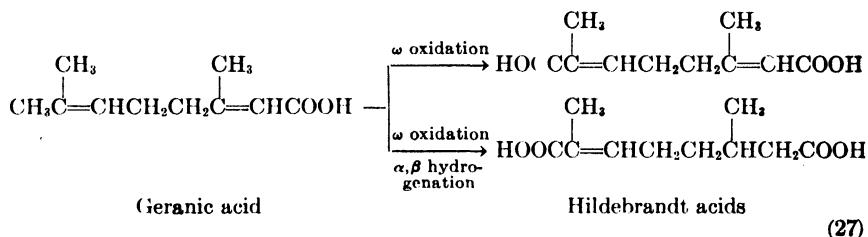
Quantitative biochemical experiments with deuterium have to be evaluated with great care, since Erlenmeyer, Schönauer, and Süllmann (90) have shown that succinic dehydrogenase acts 40% more rapidly on ordinary succinic acid than on succinic acid- d_2 . It probably does matter, therefore, as far as the spatial and ionoelectric adaptability of the substrate to the enzyme is concerned, whether an ordinary hydrogen atom or one that is twice as heavy is located at the point of reaction. Similarly Günther and Bonhoeffer (116) have

shown that ordinary hydrogen is utilized at three times the rate of deuterium in the synthesis of fats by yeast.

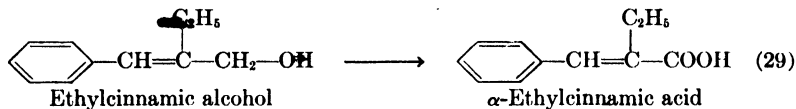
More important than α,β dehydrogenation and probably of significance in the synthesis of fatty acids in the body is the hydrogenation of the double bond in an α,β position with regard to the carboxyl



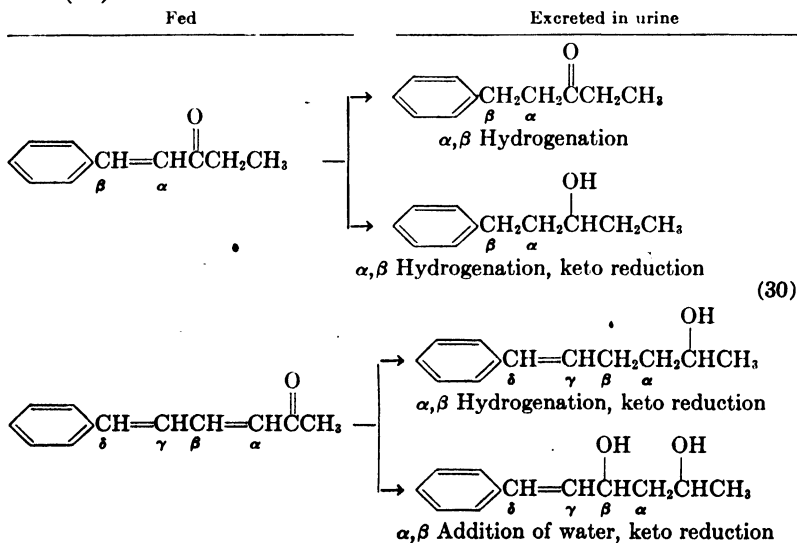
group, as of crotonobetaine (eq. 26). This was first observed by Linneweh (186). Kuhn, Koehler, and Koehler (165) were able to observe the same α,β hydrogenation when geranic acid passed through the body (eq. 27).



Fischer and co-workers (for synopsis see 99) had already observed previously with yeast that even in aldehydes and alcohols double bonds can be saturated when they are in α, β position, but not when they are farther away. Fischer and Bielig (100) have done the most important work, basically, on α, β hydrogenation in the animal. When they fed cinnamic alcohol to dogs, they found the results of reaction (28). When an ethyl group was substituted in the α position, only the hydroxyl group was oxidized to a carboxyl group (eq. 29). Keto groups exhibit the same behavior in this respect as carboxyl



groups (reactions 30). Enzymes will hydrogenate only those double bonds in unsaturated keto compounds which are in α, β position with respect to the keto group. They will not act on other double bonds. At the same time the keto group can be independently reduced to a hydroxyl group. Water can also be added to the α, β position in such a way that a β -hydroxyl group can be formed, as was the case in equation (29).

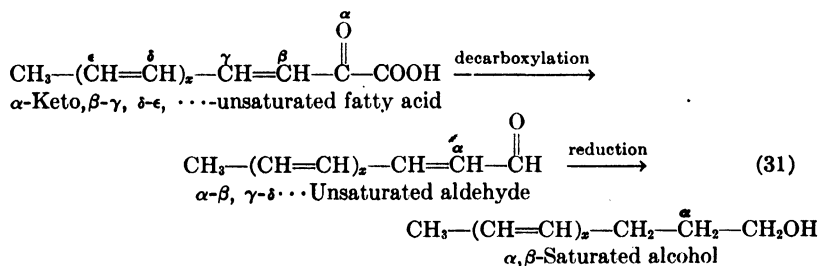


In addition to these various enzymic reactions, Fischer and Bielig also observed some oxidation at the para position of the benzene ring.

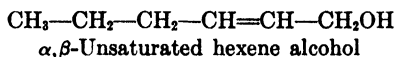
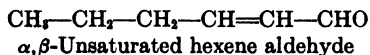
All reaction products excreted in the urine were isolated and identified by means of satisfactory methods. Beside terminal oxidation of the hydroxyl group to a carboxyl group and reduction of the keto group to a hydroxyl group, these experiments also proved that the double bonds in α, β position with respect to the carbonyl and carboxyl groups are saturated, but that mere proximity to the benzene ring does not lead to saturation of the double bond, as is shown by equation (30). Also, water can add to the double bond in α, β position with respect to the carbonyl or carboxyl group, with the result that the hydroxyl group is in β position. This addition can also be conceived of as taking place in two steps: first, saturation of the α, β double bond and then oxidation at the β carbon with the formation of the β -hydroxyl group. For this reason one cannot say that direct addition of the water molecule to the α, β double bond has been completely proved. Such proof can only be furnished if α, β -unsaturated fatty acids are subjected to short-term incubation experiments under completely anaerobic conditions, and if β -hydroxy fatty acids are isolated subsequently, at the same time excluding any β oxidation (even by way of keto acids formed as intermediates).

α, β -Unsaturated fatty acids, such as α, β -decenoic acid, are oxidized to higher methyl ketones by *Penicillium glaucum*, according to Thaler and Eisenlohr (282); probably, they are first oxidized to form higher β -keto acids (see equation 4, page 350).

Unsaturated α -ketopolyenic fatty acids are first decarboxylated by yeast (reaction 31), and the unsaturated aldehydes formed are then reduced to the corresponding alcohols saturated in the α, β position. Double bonds which are farther away from the oxygen atom are not attacked (101).



Occasionally the α, β hydrogenation is accompanied by reduction of the carboxyl group, particularly of aldehyde groups. Neuberg



(34)

According to Nye and Spoehr (221) the α,β -hexene aldehyde isolated from plant leaves is an artifact resulting during isolation from the oxidative destruction of the linoleic acid of the plant. The reason for this supposition is that the yield can be appreciably increased if linoleic acid is added while the minced plant tissue is undergoing steam distillation.

The data just presented permit us to conclude that there also exists an enzyme system which, though perhaps less important and quite unrelated to the enzyme system that brings about β oxidation of fatty acids, catalyzes α,β dehydrogenation or hydrogenation of fatty acids. The occurrence of α,β hydrogenation has been proved beyond doubt; this proof is still outstanding for α,β dehydrogenation.

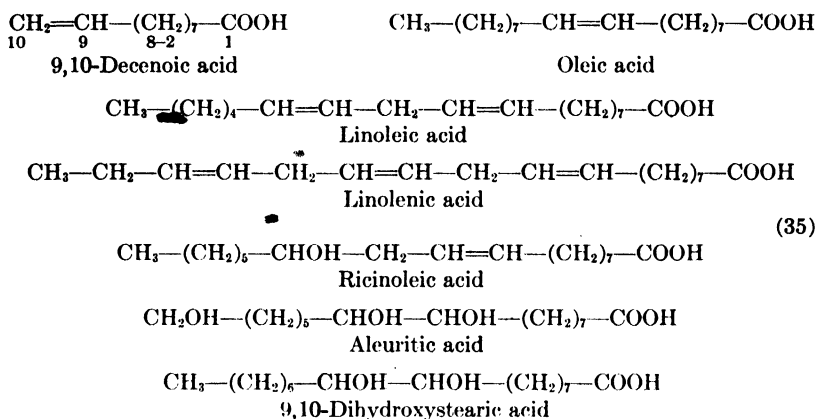
V. 9,10 Dehydrogenation

The existence of an enzyme system which dehydrogenates higher saturated fatty acids has been reported for a number of animal tissues, *e.g.*, small intestine (65a,65b), bile, pancreas (281), adipose tissue (229,247), kidneys (201), and liver (6). Annau, Eperjessy, and Felszeghy (6) report that only lecithin, palmitic acid, and stearic acid can be dehydrogenated, but that this is not the case for oleic acid. Xanthine is said to be the activator of this system. Lang and Mayer (172,173) conducted Thunberg experiments and found that saturated fatty acids with chain length of undecanoic acid (C_{11}) or more acted as hydrogen donors. By isolating the products they were able to determine that dehydrogenation takes place only between the ninth and tenth carbon atoms, counting from the carboxyl group. According to Karrer and Koenig, more highly branched fatty acids are also dehydrogenated by liver extracts (142).

The reverse reaction, *i.e.*, saturation of the 9,10 double bond, also takes place in the body. Hilditch and Pedelty (126) have shown that oleoglycerides are converted to stearglycerides by enzymic hydrogenation, while Zeller and Maschek (318) were able to isolate an enzyme from pumpkin seedlings which saturates unsaturated fatty acids.

Little is known as yet about the mechanism of 9,10 dehydrogenation, primarily because of the low stability of the enzyme preparations involved.

Almost all known unsaturated fatty acids occurring in nature have their first double bond between the ninth and tenth carbon atoms (formulas 35). This is true of oleic acid, palmitoleic acid (C_{16}), and



of multiple-unsaturated fatty acids, such as linoleic and linolenic acids. To this group belongs aleuritic acid, occurring in shellac (214,234,294) and 9,10-dihydroxystearic acid (147), which occurs in castor oil. Apparently both of these products represent biological oxidation products of a 9,10-unsaturated fatty acid. According to Appel, Böhm, Keil, and Schiller (7), even the unsaturated fatty acids C_{13} , C_{15} , C_{17} found in fat depots after ingestion of saturated fatty acids with an odd number of carbon atoms contain all their first double bonds in the 9,10 position. It is not clear whether the hemolytic, fatty acid-like material ($C_{22}H_{42}O_5$), which Laser and Friedmann (176a) isolated from serum in crystalline form should also be included in this group.

The ability of the animal organism to dehydrogenate fatty acids seems exclusively limited to the 9,10 position (in addition, perhaps, to α,β dehydrogenation). This is proved by the ability of the animal organism to produce oleic acid from stearic acid, while it is unable to produce linoleic and linolenic acids (18). Bernhard, Steinhäuser, and Bullet (19) were able to prove this by feeding deuterium oxide to animals: all fatty acids of the body contained deuterium, while the

linoleic and linolenic acids isolated did not. Linoleic acid makes up 60% of all fatty acids of the cholesterol esters found in the blood. Kelsey and Longenecker (146) isolated these, after Huerthle (133) had already observed in 1896 that the cholesterol esters in blood contained highly unsaturated fatty acids. Both linoleic and linolenic acids possess vitamin-like characteristics. Their biochemical role in the body is still unknown. They have to be supplied the animal body from the plant world, as only plants are capable of synthesizing them (53). For the subject of essential fatty acids, see Bernhard (15b).

In the case of naturally occurring simple unsaturated fatty acids of medium chain length, the first double bond is also found in the 9,10 position. Smedley (253) was the first to determine the presence of 9,10-decenoic acid (formulas 35) in butter fat, and Bosworth and Brown (33) isolated the acid. Hilditch and Longenecker then showed in 1938 that butter fat also contains 0.9% 9,10-dodecenoic acid, 1.7% 9,10-tetradecenoic acid, 3.7% 9,10-hexadecenoic acid, and palmitoleic acid, in addition to 0.4% 9,10-decenoic acid (125).

An exception to the 9,10 rule in the animal body is arachidonic acid, containing four double bonds, $\Delta^{5,8,9,11,12,14,15}$ -eicosatetraenoic acid. A few other rare acids of similar structure also are an exception to this rule. Section VIII contains a discussion concerning their possible origin. According to Turpeinen (296) and Hume, Nunn, Smedley-Maclean, and Smith (134), arachidonic acid, like linoleic and linolenic acids, possesses vitamin F-like characteristics. If, however, the carbon chain of linolenic acid is lengthened synthetically by means of the Arndt-Eistert method of adding one or two CH_2 groups behind the carboxyl group, the acid will no longer act as a vitamin, according to Karrer and Koenig (see 143). The vitamin function, in other words, is dependent on the first double bond in the 9,10 position.

Unsaturated acids which have their double bond in a different position occur in plant oils. Nevertheless, the great majority of unsaturated acids occurring in plant oils also have their first double bond in the 9,10 position (208).

In view of the known facts it is unlikely that reversible dehydrogenation at the 9,10 position of the chain represents one of the mechanisms in the enzymic degradation of fatty acids. If further oxidation were to take place at the double bond, with resultant splitting

of the chain, we would expect that the body splits oleic acid to the difficultly oxidizable azelaic acid (C_9 -dicarboxylic acid) and to nonoic acid. Verkade and van der Lee (299) attempted to prove this catabolic mechanism by feeding triolein, but were unable to isolate azelaic acid from the urine; similarly, the formation of nonoic acid, which in turn would also form azelaic acid after ω oxidation, has never been observed.

It seems much more reasonable to assume that the body dehydrogenates saturated fatty acids, since they have a high melting point, at the 9,10 position, in order to obtain more liquid unsaturated fatty acids which have a low melting point and therefore offer a way for the body to keep its fat at the semisolid consistency which moving tissues require. This point of view is supported by work done by Henriques and Hansen (123), who investigated the melting point and, as a measure of unsaturation, the iodine number of the fat layers of hogs. These values were determined as a function of the distance from the skin, that is, in layers which differ from one another in their temperature, because of the contrast between the cold outer air and the warm interior of the body. Their results are shown in (36).

Fat layer	Iodine number	Melting point of fat, $^{\circ}C$.	(36)
Most exterior	72	22	
Second	70	24	
Third	65	25	
Fourth	64	25	
Internal depot fat	56	29	

Experiments by André (3) have shown, however, that desaturation is not the only means available to the animal organism for adapting the melting point of its fats to the prevailing temperature conditions. He found that the outer layer of the heavy fat layer of marine animals does not have a higher iodine number, as was the case with hogs, but that it, on the other hand, contains a larger number of low-melting glycerides of lower fatty acids, such as glyceroltripentanoic acid ester.

We can conclude that the enzyme system involved in the dehydrogenation of fatty acids at the 9,10 position probably has nothing to do with the catabolism of fatty acids; rather it probably plays a role in the mechanism available to the cell for the control of the physical consistency of

the body fats. Dehydrogenation may also have the function of rendering saturated fatty acids more readily diffusible, as their sodium salts diffuse with difficulty and thus are not readily resorbed. Sodium oleate is more readily diffusible and soluble than sodium stearate.

VI. Lipoxidases

Although it can be considered certain that there is no degradation of fatty acids which proceeds by way of splitting the double bond of a fatty acid, as was assumed by Leathes, there nevertheless do exist specific enzymes, the so-called lipoxidases, which attack fatty acids with several double bonds (4). These enzymes are found primarily in plant seeds, particularly in legumes (272), in potato tubers (273), and to some extent in animal tissues (33,177). This last finding has been questioned, since Kuhn and Meyer (168) observed that ordinary iron porphyrins catalyze the oxidation of multiply unsaturated fatty acids without an enzyme system. Haurowitz, Schwerin, and Yenson (122) observed that pure crystallized hemoglobin, which is present in all animal tissues in large quantities, acts as a good catalyst in the direct oxidation of doubly unsaturated fatty acids by oxygen; this is even true of hemin, which contains no protein. In this reaction, the porphyrin ring itself undergoes peroxidative destruction. Linoleic acid cholesterol esters, which occur in the blood and are vital vitamin-like substances, apparently are protected against oxidation by blood hemoglobin. Cosby and Sumner (65) were able to isolate lipoxidase in sixtyfold concentration. Theorell, Holman, and Åkeson (283b) were able to obtain crystallized lipoxidase by concentrating soybean meal 150-fold. They recovered a pure protein, containing no metal components.

The substrates of lipoxidases are higher unsaturated fatty acids, like linoleic and linolenic acids, or carotenoids. Saturated fatty acids and oleic and ricinoleic acids, which have an unsaturated double bond in the 9,10 position, are hardly attacked at all (273).

According to Meyerhof (207) and Hopkins (130), thiol compounds, such as glutathione, greatly accelerate these oxidations, but only when the pH is acid. If carotene is added to a lipoxidase-linoleic acid mixture, it is secondarily destroyed as a result of the action of the peroxidative reaction products (276). In this process, linoleic acid adds 1 mole of O_2 to one of its bonds (275), perhaps the 9,10

double bond. It is interesting to observe that linoleic and linolenic acids which have conjugated double bonds cannot serve as the substrate for lipoxidase, but that normal linoleic acid is required, in which the double bonds are separated by a CH_2 group (129; equation 35). According to Holman and Burr (129) and Sällman (274), the reaction products resulting from the action of lipoxidase on highly unsaturated fatty acids are ketones and polymer-like products; this has been proved by the fact that nonidentifiable phenylhydrazones and semicarbazides have been isolated. It may be that aleuritic acid which occurs in shellac (eq. 35) is a result of similar reactions. The mechanism of lipoxidase action is discussed in a separate article in this volume by Bergström and Holman.

Since fatty acids like linoleic acid, on which these oxidases act, have a vitamin-like character, *i.e.*, cannot be synthesized by the animal body, it can be concluded that the lipoxidases play no role in the fatty acid catabolism of animal cells; these acids therefore cannot represent an intermediary step in the catabolism of the fatty acids.

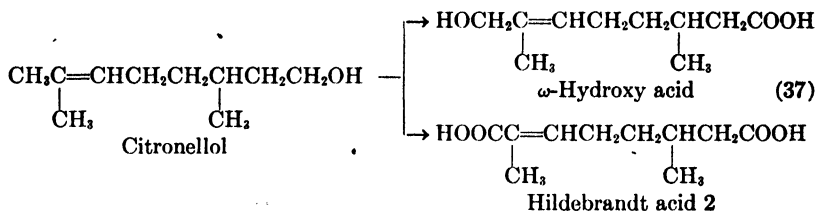
We therefore can say that the lipoxidases which occur primarily in plants play no role in the mechanism of the chemical degradation of biochemical catabolism of fatty acids by the animal organism.

VII. ω Oxidation of Fatty Acids of Medium Chain Length

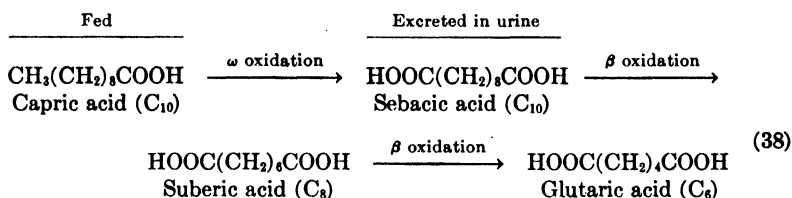
The earliest known publication on ω oxidation is that by Hildebrandt (124), who administered geraniol and geranic acid and then isolated two similar dicarboxylic acids in the urine (see equation 27, page 365). He concluded erroneously that the β -methyl group had been oxidized to a carboxyl group. Only Kuhn and Livada (167) determined that it really is the terminal methyl group which is oxidized to a carboxyl group in this reaction. According to Kuhn, Koehler, and Koehler (165), the Hildebrandt acids differ from one another in that the α,β double bond of one is saturated. If the constitution of the acid found in 1901 had been properly interpreted at that time, ω oxidation would have been discovered thirty years earlier.

The formation of the corresponding ω alcohol seems to be the intermediary step of ω -methyl oxidation. Fischer and Bielig (100), after feeding citronellol isolated both the partially racemized Hildebrandt acid 2 and the corresponding alcoholic acid (see eq. 37).

In 1932 Verkade and van der Lee (297) discovered that the triglycerides of saturated fatty acids of medium chain length, *i.e.*, those



having between eight and eleven carbon atoms, were partially excreted in the urine as the corresponding fatty dicarboxylic acids (eq. 38). They called this process ω oxidation.



In feeding experiments conducted on themselves, Verkade and van der Lee (298) found the following:

Ingested	Excreted
100 g. trinonylin.....	0.6 g. azelaic acid (C_9)
25 g. tricaprin.....	0.55 g. sebacic acid (C_{10})
25 g. triundecylin.....	1.6 g. undecanedioic acid (C_{11})
25 g. trilaurin.....	Few mg. dodecanedioic acid (C_{12})

Flaschenträger had already observed in 1926 (102) that when sebacic acid, $\text{HOOC}-(\text{CH}_2)_8-\text{COOH}$, was ingested, part of it was excreted unchanged in the urine, while part of it was excreted in the form of suberic acid, $\text{HOOC}-(\text{CH}_2)_6-\text{COOH}$, i.e., sebacic acid had lost two carbons by β oxidation. When azelaic acid was fed, $\text{HOOC}-(\text{CH}_2)_7-\text{COOH}$, 60% of it was excreted unchanged (254). Experiments by Verkade and van der Lee (298), Verkade, van der Lee, and Alphen (300), and Bernhard and Andreae (16) have shown with fairly good agreement that, when 10 g. adipic acid is fed, 18% is excreted unchanged, when 10 g. suberic acid is fed, 60 to 80% is excreted in the urine unchanged, and, when sebacic acid is fed, about 20% is excreted unchanged. Verkade and van der Lee (299) used these findings to demonstrate that there probably is no relation between the 9,10 dehydrogenation of fatty acids and fatty acid catab-

olism, since no trace of the expected azelaic acid was found in the urine after triolein had been fed in large quantities.

Fatty dicarboxylic acids behave quite differently if one of the carboxyl groups is blocked. Thus Flaschenträger (102) reports that sebatic semiamide *i.e.*, sebatic acid, one carboxyl group of which has been changed to an amide group and therefore is probably inactive biochemically, is 95% oxidized. Similarly Flaschenträger and Bernhard (104) and Bernhard (12) report that the semiethyl esters of dicarboxylic acids having eight to ten carbon atoms are oxidized to a greater extent in the body than the free dicarboxylic acids. Semi-methyl- and propylamides are oxidized completely (15):

Fed	Excreted in urine (remainder oxidized)
Sebacic acid, $\text{HOOC}(\text{CH}_2)_8\text{COOH}$	ca. 50%
Sebacic semiethyl ester, $\text{HOOC}(\text{CH}_2)_8\text{COOC}_2\text{H}_5$	ca. 8% (39)
Sebacic semiamide, $\text{HOOC}(\text{CH}_2)_8\text{CONH}_2$	ca. 5%
Sebacic semimethylamide, $\text{HOOC}(\text{CH}_2)_8\text{CONHCH}_3$	0

These experiments thus permit us to conclude that the reason dicarboxylic acids of eight to ten carbon atoms are catabolized with difficulty is that the second carboxyl group interferes sterically with the action of the enzyme system necessary for β oxidation of the first carboxyl group. If, however, the second group has been blocked as an amide or an ester, the fatty acid can be broken up more rapidly by means of β oxidation proceeding from the free carboxyl group than hydrolyzing enzymes are able to change the blocked amide or ester group back to a free carboxyl group with steric hindrance.

Terminal methyl oxidation, ω oxidation, also occurs among the carboxylamides of fatty acids having four to six carbons, if they have double bonds (166). In this case the amides behave differently from the amides of the saturated acids. Thus, while sorbic acid, $\text{HOOC}-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{CH}_3$, is completely oxidized by the body, its amide yields 32% muconic acid, $\text{HOOC}-\text{CH}=\text{CH}-\text{CH}=\text{CH}-\text{COOH}$, as Kuhn, Koehler, and Koehler found (166).

When polyenic carboxylic acid amides were ingested, the yields of excreted dicarboxyl acids given in scheme (40) were obtained. It

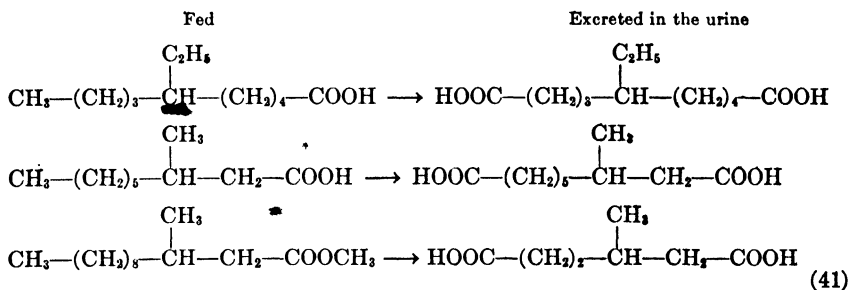
Fed	(40)	Isolated from urine	Amount
$\text{CH}_3-\text{CH}=\text{CH}-\text{CONH}_2$		None	
$\text{CH}_3-(\text{CH}=\text{CH})_2-\text{CONH}_2$	→	$\text{HOOC}-(\text{CH}=\text{CH})_2-\text{COOH}$	32%
$\text{CH}_3-(\text{CH}=\text{CH})_3-\text{CONH}_2$	→	$\text{HOOC}-(\text{CH}=\text{CH})_3-\text{COOH}$	42%
$\text{CH}_3-(\text{CH}=\text{CH})_4-\text{CONH}_2$	→	$\text{HOOC}-(\text{CH}=\text{CH})_4-\text{COOH}$	20%

may be that the percentage yields of the higher acids are out of proportion because of their low solubility and the consequently difficult resorption capacity. Dimethylacrylic amide, $\text{CH}_3\text{—C}(\text{CH}_3)=\text{CH—CONH}_2$, yields 43% *trans*- $\text{HOOC—C}(\text{CH}_3)=\text{CH—COOH}$; $\text{CH}_3\text{—CH}_2\text{—C}(\text{CH}_3)=\text{CH—CONH}_2$ apparently does not undergo ω oxidation at all, while $\text{CH}_3\text{—}(\text{CH}_2)_2\text{—C}(\text{CH}_3)=\text{CH—CONH}_2$ is only 13% ω oxidized. We thus see that when a carboxyl group is blocked in the form of an amide, ω oxidation takes place more rapidly than does the more normal β oxidation, even though the fatty acid has a shorter chain.

When dicarboxylic acids are formed in the body from alkylated branched fatty acids, a phenomenon takes place which Linke (185) designated as the carboxyl effect. While on the one hand terminal methyl groups are relatively easily oxidized, methyl groups in α position will not be oxidized to a carboxyl group by β oxidation. The adjoining carboxyl group prevents this oxidation; in other words, substituted malonic acid is not formed at any stage of intermediary metabolism. This is the reason that propanoic acid (α -methylacetic acid) is the only fatty acid known which undergoes α oxidation and probably is oxidized to δ -lactic acid (29,114,237; see, however, 153). So far as is known, propanoic acid does not undergo β oxidation. Lactic acid may, however, undergo β oxidation in spite of the above facts; this is supported by the findings of von Euler and Bolin (91), who found mesoxalic acid in *Medicago sativa*, in addition to glycolic and glyoxylic acids. It should be added that mesoxalic acid may also be formed by some other method, such as from glyceric acid.

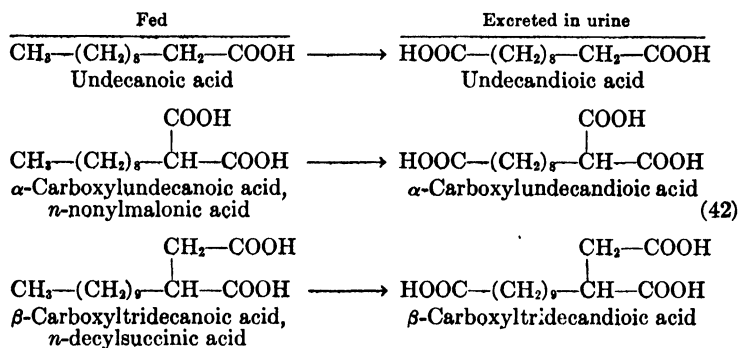
Alkyl side chains, on the other hand, whether they are in α, β, γ , or δ position or not, seem to exert a partial hindrance on β oxidation. This may be due to the fact that the molecules are widely branched and thus make it more difficult for active enzyme groups to approach the molecule. This may also be the reason that terminal methyl oxidation, which as such is potentially weaker, but which may have less steric interference to contend with from the side chains, is capable of oxidizing branched fatty acid molecules more readily to dicarboxylic acids (reactions 41), particularly if the main chain is only of medium length. Thus Keil (144) reports that ϵ -ethyl- C_{10} acid gave a high yield of ϵ -ethylsebacic acid, while unbranched C_{10} acid, when fed under similar conditions, gave a much smaller yield of sebacic acid. Ethyl- and especially propyl-branched fatty acids are

partly excreted unchanged (144a). Branched unsaturated fatty acids like geranic acid if they are in the form of amides, can readily be made to undergo ω oxidation with good yields. When it is desired to prepare some of these dicarboxylic acids, this biochemical oxidation by the animal body often is the only available method of preparation, or at least the only one with good yields (165,166).



A paper by Thomas and Weitzel (288) also supports the suggestion that branched fatty acids form dicarboxylic acids with relatively great ease. They found that when they fed synthetic fat which had been prepared from paraffins obtained through the Fischer-Tropsch-Gatsch process ($\text{CO} + \text{H}_2$), the urine contained liquid branched dicarboxylic acids. These fats contained 15–20% of branched fatty acids (183,288a). Fats with unbranched chains which had been prepared from hydrogenated lignite paraffins yielded no measurable quantities of dicarboxylic acids under similar conditions. Here too the branched fatty acids are diacidogenic, while unbranched ones are not (see also reference 7).

A compilation of all known data concerning methyl oxidation will be found in the paper by Linke (185). Emmerich, Neumann, and Emmerich-Glaser (87) fed low alkylated succinic acids; Bernhard and Linke (17) fed high alkylated acids (see also 288b); they found succinic acid in part and unchanged alkylsuccinic acid in the urine. 29% of the β -carboxytridecanoic acid was ω oxidized and excreted as β -carboxytridecanedioic acid. The corresponding dodecanoic and pentadecanoic acids did not undergo ω oxidation. The introduction of one carboxyl group in the β position thus shifts the maximum of ω oxidation from a chain length of C_7 – C_{11} to one of C_{13} . If we consider alkylated succinic acid, however, as a normal fatty acid with an acetic acid group in α position, the maximum is at C_{12} .



Similar results were obtained in feeding experiments of Bernhard (14) with alkylated malonic acids. Nonylmalonic acid gave the maximum excretion of all dicarboxylic acids, namely, 8.8% tricarboxylic acid. The maximum here too, in other words, was reached when the chain length was C_{11} . According to Emmerich and co-workers, dimethyl- and dibutylmalonic acids were excreted unchanged for the most part; Bodur reports the same (31) for symmetrical, and Weitzel (308a) for asymmetrical dimethylsuccinic acid. See Linke (185) for a list of the specific reports.

The fact that deviations tend to occur among the fatty acids of medium length even in different reactions is also shown by the observation of Breusch and Tulus (44). They found that, when oxaloacetic acid condensed in pigeon muscle in the presence of β -hydroxy fatty acids, the C_9 and C_{10} β -hydroxy acids resulted in a particularly low yield of "citric acid," while the lower β -hydroxy acid homolog, *i.e.*, C_8 , and the higher homologs, C_{11} , C_{12} , C_{13} , and C_{14} , gave much higher yields.

That ω oxidation has no connection with normal fatty acid oxidation was directly proved by Bernhard (15a). Fed azelainic acid (C_9) is mostly excreted in the urine. A deuterium-labeled azelainic acid should therefore have a decreased deuterium content if it is diluted in the body by normal azelainic acid derived from normal fatty acids by 9,10 dehydrogenation, splitting of the chain, and subsequent ω oxidation. Experimentally, the deuterium content of the excreted acid was exactly the same as that of the acids ingested. Thus, no dicarboxylic fatty acids are formed in normal metabolism, and neither ω oxidation nor a catabolic breakdown of the fatty acid chain between the ninth and tenth carbon atoms takes place.

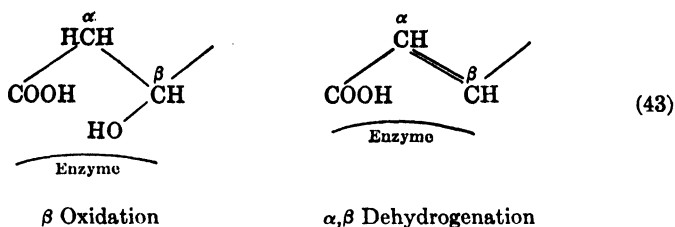
ω Oxidation, the methyl oxidation of paraffin end of the fatty acid molecule, is not a general catabolic mechanism for fatty acids, but is restricted to fatty acids of medium chain length, C_8 to C_{11} . In these acids ω competes with β oxidation. Flaschenträger and Bernhard (see 103) estimate that 90% of a given acid of this chain length undergoes β oxidation, while 10% undergoes ω oxidation. It is not known where in the body ω oxidation takes place. Since the principal route of fatty acid degradation appears to be alternating β oxidation in the liver, fatty acid molecules of medium chain length probably are not formed at all or only to a small extent in the process of catabolism in the animal body, with the result that ω oxidation has no significance in the normal metabolism of fatty acids.

VIII. Attempt to Formulate a Comprehensive Theory Explaining the Known Mechanisms of Enzyme Action on Fatty Acids

When considering the 4 known mechanisms of biochemical action on fatty acids, it will be noted that two of the mechanisms, β oxidation and α,β dehydrogenation, are concerned with the second and third carbon atoms of the fatty acid chain, while the other two mechanisms, ω oxidation and 9,10 dehydrogenation, are concerned with reactions at the ninth and tenth carbon atoms. There can be no question that β oxidation and α,β dehydrogenation should be considered together, even though two different enzyme systems are involved. It can be assumed that 9,10 dehydrogenation and ω oxidation also belong together.

If an attempt is to be made to set up a theory which for purposes of a more unified understanding assumes that all reactions involved in the metabolism of the fatty acid chain can be reduced to one or more similar mechanisms, it will be necessary to be able to construct such spatial arrangements of the fatty acid chain that the 2,3 (the α,β) and the 9,10 carbon atoms can be brought into similar positions with respect to the active enzyme group. If we make the probable assumption that the fatty acid molecule is attached to the enzyme protein by its carboxyl group only, we obtain the following structural picture shown in equation (43).

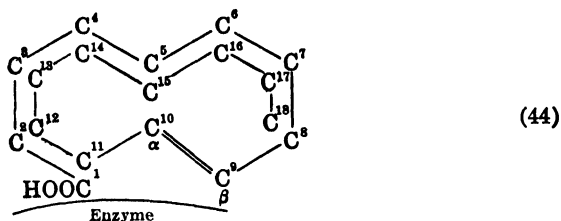
β Oxidation and α,β dehydrogenation can be spatially understood, since the point of attachment and the point of reaction of the fatty acid molecule are so close to one another on the enzyme protein mole-



cule that a chemical reaction can take place, even though the paraffin chain can move and rotate quite readily.

The specificity of the dehydrogenation and ω oxidation enzyme systems for the ninth and tenth carbon atoms, on the other hand, is quite incomprehensible. The reason for the difficulty lies in the softness and flexibility of the paraffin chain which are so great that it is impossible to produce even antigens for fatty acid residues which have been chemically introduced into protein surfaces (120,170); this can usually be done with any chemical group of sufficient rigidity to leave a definite impression on the surface negative of the antigen (121).

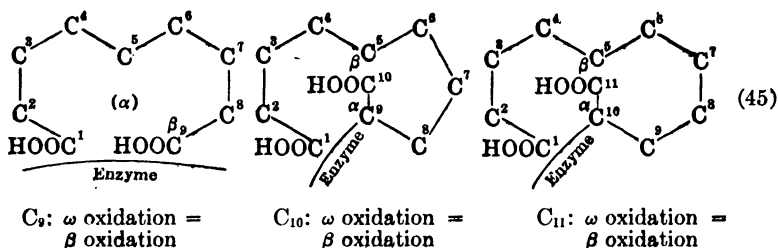
The specificity of the enzyme for the ninth and tenth carbon atoms can only be explained if we assume that that part of the paraffin chain which is behind the carboxyl group assumes a definite shape; this shape must be a ring system the spatial angles of which must be in agreement with those of the bonds between the carbon atoms. The system has to be so rigid that the chain is in an absolute position making it possible for 9,10 dehydrogenation to occur, since no double bonds are found in naturally occurring fatty acids that are closer to the carboxyl group (except for arachidonic acid and some other homologous acids which occur rarely). In order for ω oxidation to occur, the eighth and eleventh carbon atoms must also be in a position to react, although to a lesser extent than was required of the ninth and tenth carbon atoms. The reason for this requirement is the fact that fatty acids of this chain length have been shown to undergo ω oxidation. In the case of ω oxidation we must be able to find a position which will be more elastic than was the case for 9,10 dehydrogenation. These requirements are best met by an arrangement where the chain is in the form of a ten-membered ring. If the molecule has more than ten carbon atoms, the ensuing carbon atoms will arrange themselves concentrically around the first ten carbon atoms, somewhat in the manner in which crystals form.



9,10 dehydrogenation = α,β dehydrogenation

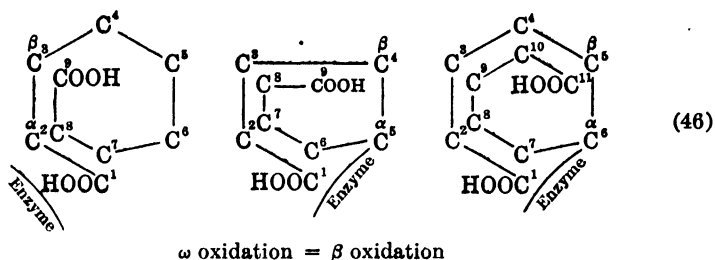
If the elements in excess of ten do crystallize onto the ring, the 9,10 position would indeed be adequately fixed in place. In this fixed position the α,β position has become identical with the 9,10 position and the same dehydrogenation would occur in both instances. This does not mean, however, that the same enzyme system is involved in both reactions.

The situation is different in the case of ω oxidation. Those fatty acids which are primarily ω -oxidized, *i.e.*, the C_9 , C_{10} , and C_{11} acids, have no more carbons available, once they have formed the ten-membered ring, with the result that the chain no longer is so rigid as is the case with the higher fatty acids.

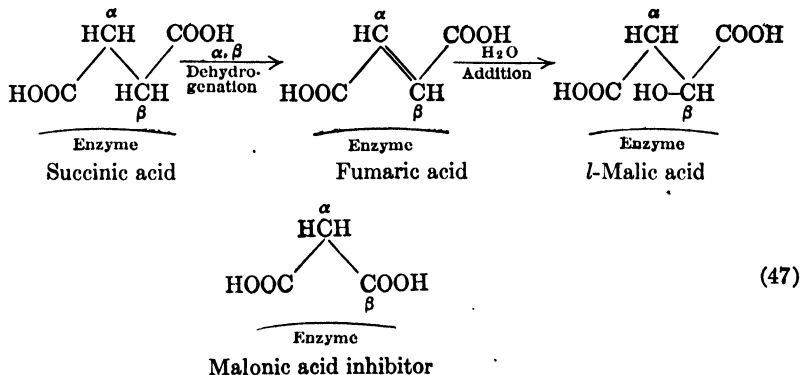


The chain is less rigid, thus permitting in each case a steric arrangement which is consistent with the allowed valence angle and yet permits the terminal methyl groups to be in β position with respect to the carboxyl group; hence ω oxidation can occur. Such ω oxidation then would be identical with β oxidation, even though the enzymes involved are not the same ones.

Spatially the following arrangement is also possible, *i.e.*, one in which C_9 - C_{11} fatty acids are fixed in place by intramolecular folding over the chain.

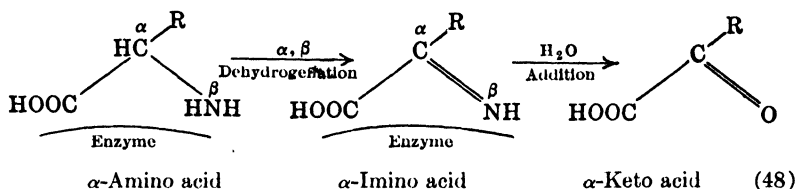


The sidetracking of β oxidation in favor of ω oxidation which occurs after heavy administration of C_9 , C_{10} , and C_{11} fatty acids may be a result of stronger attachment to the protein of the second ω -carboxyl group just formed than is the case for an ordinary β -hydroxy or β -keto group which may have been formed in the course of β oxidation. Thus the substrate may remain attached to the enzyme for a longer period, preventing the enzyme from becoming active in the actual β oxidation. In this connection, attention is called to the papers of Flaschenträger and Bernhard (104) and Bernhard (15), concerned with ω oxidation of sebacic acid, which were discussed previously. They blocked one of the carboxyl groups by esterification or amidation and found that the compounds then were oxidized more readily than originally. This may also explain why malonic acid, a β -carboxylated acetic acid, specifically interferes with the α, β desaturation of succinic acid to fumaric acid, and thus with its oxidation to *l*-malic acid:

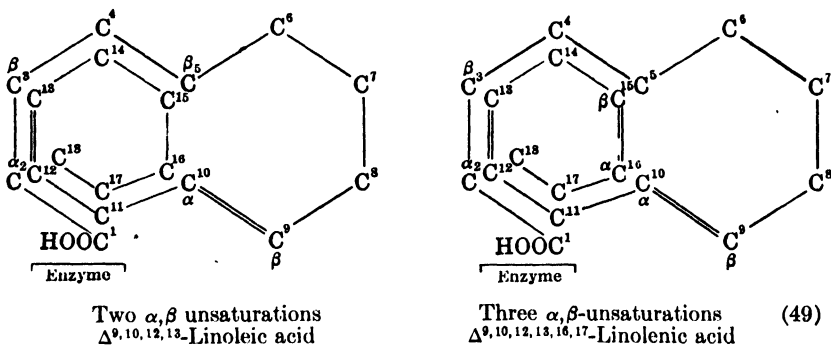


This suggestion leads us to the remarkable conclusion that the mechanism of succinic dehydrogenase action is similar to that of

α, β dehydrogenation and β oxidation. Thus we can ascribe ω oxidation to a partial poisoning of the enzyme surfaces which are needed for α, β dehydrogenation and β oxidation. The poisoning is effected by C_9 , C_{10} , and C_{11} dicarboxylic acids which are similar in structure to malonic acid (see equation 45). These in turn, although formed in small quantities, stimulate further ω oxidation of the C_9 , C_{10} and C_{11} fatty acids. This steric explanation of a hindered α, β dehydrogenation also applies to the oxidative dehydrogenation of the amino acids. Thus, 0.03 M malonic acid stops deamination in rat livers almost completely (57).

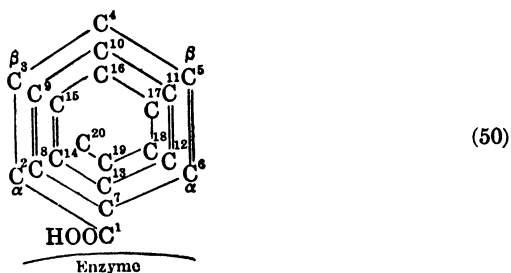


This formulation has further uses. Almost all unsaturated fatty acids which occur in nature and which have two or three double bonds do not have conjugated double bonds, but double bonds which are separated from one another by CH_2 groups. The scheme given for oleic acid can be developed to show that every double bond originates from an α, β dehydrogenation.



Our assumption that the ten-membered rings which we have suggested are stable is based on experimental results of Hückel, Gerke, Gross (132) and Plattner and Hulstkamp (225). They found that derivatives of cyclodecane, like 1,5-dioximes, readily form derivatives

of decahydronaphthalene, decalin, by closing the ring. We thus see that the suggestion of a ten-membered ring as postulated may actually correspond to the conditions prevailing in the molecule. The fact that the distillation of barium and thorium salts of undecanedioic acid (C_{11}) results, according to Ziegler and Aurnhammer (320), in a low yield of cyclodecanones does not contradict the assumption of a concentric cyclodecane-like ring which has been made here, since in ω oxidation only one end has a carboxyl group, while the other had a paraffin chain. This theory, if slightly varied, can also explain the origin of arachidonic acid. According to Dolby and Nunn (73) and Movry (211), arachidonic acid is $\Delta^{5,6,8,9,11,12,14,15}$ -eicosatetraenoic acid (C_{20}). Its structure can be thought of as originating by α, β dehydrogenation in the following manner:



α, β Dehydrogenation occurring four times
 $\Delta^{5,6,8,9,11,12,14,15}$ -Eicosatetraenoic acid (arachidonic acid)

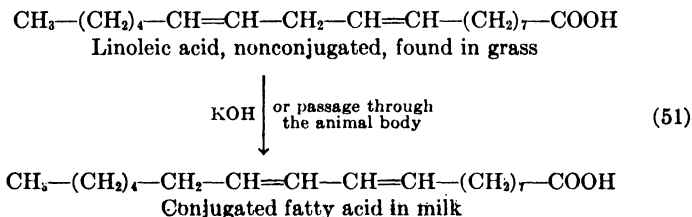
Clupanodonic acid, which has five double bonds and which occurs in the phosphatides of the brain, accordingly would have the structure of a $\Delta^{5,6,8,9,11,12,14,15,17,18}$ -pentaenoic C_{20} acid, since there is room for another double bond in the structure indicated above, if we except the 2,3 position.

Further indication that the 9,10 position is specific for biochemical reactions based on spatial arrangements along the surface of the enzyme can be seen by linoleic acid's loss of vitamin F characteristics if its chain is synthetically lengthened by 1 or 2 CH_2 groups (143). Even a shift in the position of the double bond from the 9,10 to the 10,11 position will cause a loss of the vitamin characteristics. The special position held by fatty acids of medium chain length, both from a physical and biochemical point of view, is also shown by the experiments of Roelke and Reichel (239), who showed that the bactericidal

action of soaps of different fatty acids is at a maximum for chains having eight to twelve carbon atoms.

This theory will not explain the position of the double bonds in the multiply unsaturated fatty acids, C_{18} to C_{22} , which Toyama, Tsujimoto, and Tsuchiya (293) isolated from fish oils and which, according to the authors, have conjugated double bonds in positions 4,5 and 6,7. The observations of Steger and van Loon (265) and of Moore (209), however, who state that the customary method of saponifying fats by cooking with alkalis results in conjugation of the nonconjugated double bonds of linoleic and linolenic acids, should caution us with regard to reports on the primary position of conjugated double bonds in fatty acids.

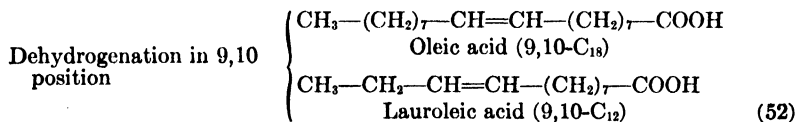
We also should remember that a secondary transformation of multiple unsaturated nonconjugated fatty acids can take place, changing them to compounds with conjugated double bonds. Thus Houston, Cotton, Kon, and Moore (131) found that fatty acids that have several conjugated double bonds (eq. 51) occur in cow's milk,



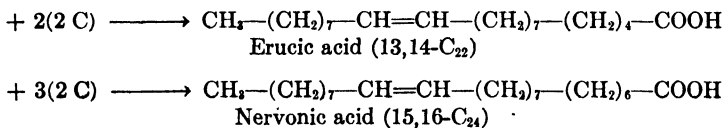
even though the multiple unsaturated fatty acids from which these were derived and which occur in the ingested grass did not contain conjugated double bonds. Since the animal body does not synthesize multiply unsaturated fatty acids, undoubtedly a secondary shift of the nonconjugated double to conjugated double bonds occurs, as the multiply unsaturated fatty acid passes through the animal body.

Thus, except for Japanese reports which still await confirmation, nearly all double bonds found in naturally occurring fatty acids start at an odd carbon, counting from the carboxyl group. Most first double bonds are either 5,6, 9,10, 11,12, or 13,14 double bonds, counting from the carboxyl group, that is, they are spaced at distances of two carbons (32). This then permits the assumption that all first double bonds were originally formed at the 9,10 position, and that the fatty acid subsequently either underwent a shortening of its chain by

(2 C)_n through β oxidation, or that its chain was increased by (2 C)_n through biosynthesis (scheme 52). This assumption then would also explain the existence of erucic acid ($\Delta^{13,14}$ -unsaturated C₂₂ acid) and nervonic acid (151) (15,16-unsaturated C₂₄ acid). They probably are a result of a subsequent biosynthetic increase in the chain of oleic acid, since oxidizing off 2(2 C) from erucic acid or 3(2 C) from nervonic acid will give $\Delta^{9,11}$ -oleic acid.



Subsequent biosynthesis



The theory presented here is as yet unproved and perhaps is more of a working hypothesis than an accurate description of what takes place. It permits us, however, to integrate into one picture not only all known mechanisms of enzyme action on fats but also the structures of almost all known unsaturated fatty acids. It is presented in order to arouse comment. Whether it is correct or will be replaced by a better theory can only be decided by further experimentation.

A structural theory is proposed which permits ascribing the most important processes of enzyme action on fatty acids, i.e., β oxidation, α,β dehydrogenation, ω oxidation, and 9,10 dehydrogenation, to the same mechanism. This hypothesis is advanced with the object of inviting criticism and comment.

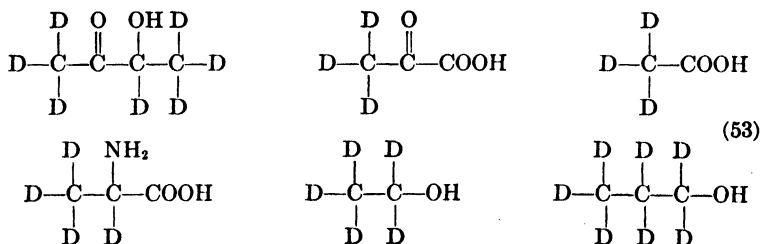
IX. Biochemistry of the Lower Fatty Acids

The metabolism of the lower fatty acids containing two to six carbons can be included in the metabolism of fat only to a limited extent, since the terminal metabolism of other food components is included therein. However, since these acids may represent fragments resulting from the catabolism of higher fatty acids, they should be considered within the framework of this review.

α -Amino acids become α -imino acids by α,β dehydrogenation and become α -keto acids through hydrolysis. The latter in turn are re-

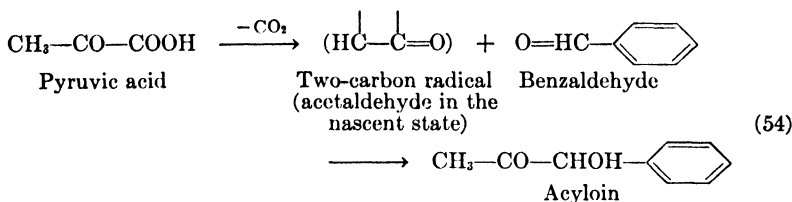
duced to α -hydroxy acids. An α -keto acid, pyruvic acid, is an intermediate step in the oxidation of glucose, lactic acid being reversibly formed from pyruvic acid. Yeast and other lower organisms can split α -keto acids anaerobically, forming aldehyde and carbon dioxide eventually. In the higher organisms, this ability to degrade α -keto acids is developed to a much more limited degree and restricted to the lower α -keto acids, *i.e.*, those having no more than four carbon atoms (112,113). In addition, warm-blooded animals are also capable of aerobically converting pyruvic acid to acetic acid and carbon dioxide. This was proved by Swendseid, Barnes, Hemingway, and Nier (279) who, after feeding pyruvic acid containing deuterium, were able to isolate from the urine acetylamine which contained deuterium in the acetyl groups (15c). This acetylation can also be proved directly by feeding "tagged" acetic acid, which is also partially oxidized (50). Acetic acid may also be oxidized at the methyl group (Buchanan, personal communication). *Aspergillus niger* grown on calcium acetate produces glycolic and glyoxylic acids (59). Nord and Vitucci (220a) have shown that wood-destroying molds transform acetic acid to succinic acid, glyoxylic acid, and oxalic acid.

The *in vitro* acetylation of amines, which takes place when pyruvic acid is catabolized, is increased by the addition of vitamin B₁ (thiamin), and is decreased by the addition of C₄-dicarboxylic acids, or by the addition of aldehydes (277). According to Bernhard, deuterium-containing acetic acid is formed after ethanol or alanine containing deuterium has been administered (15d,19a). It is also formed from *n*-butanoic acid, *n*-pentanoic acid, and even from *n*-tetradecanoic (myristic) acid, but surprisingly not from *n*-propanoic acid (26). Thus acetic acid, or any other two-carbon fragments which acts as a radical, may be the catabolic product of all food constituents. For instance, deuterium-containing acetylamines may be excreted in the urine after any of the compounds in (53) are fed.

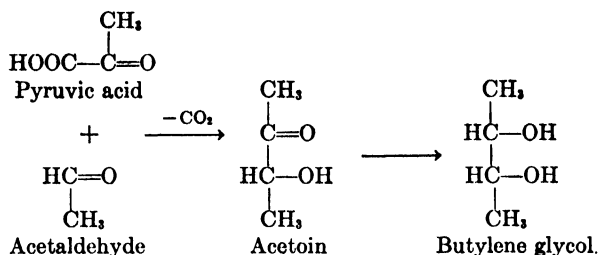


From the dilution of fed C^{13} -containing acetic acid (calc. from the C^{13} concentration of acetylaminos excreted in the urine) Bloch and Rittenberg (26) estimate that 15 to 20 millimoles (or 0.9 to 1.2 g.) acetic acid per 100 g. body weight are formed daily in the body of warm-blooded animals as catabolic products of the food eaten. This would mean that a man weighing 70 kg. would daily form about 700 g. acetic acid or other two-carbon fragments in intermediary metabolism. In other words, almost all of the nutrients which are burned per day could pass through this catabolic state.

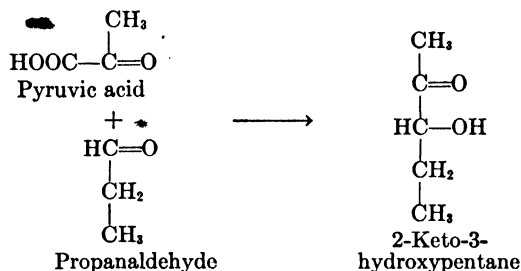
In 1921 Neuberg and Hirsch (217) discovered acyloin condensation in yeast. They found that acetaldehyde, while in the nascent state, can condense with other aldehydes, such as benzaldehyde, forming acyloins (eq. 54). Later the enzymic character of this reaction was contested (72), but apparently not successfully (169).



Pyruvic acid and acetaldehyde (perhaps acetic acid too) can also biochemically undergo the condensation to acetoin (eq. 55) or diacetyl, according to Hirsch (126b); also in butyric acid bacteria, according to Virtanen, Kontia, and Storgards (301). This kind of condensation can also take place in animal tissues (113). Martius (197) suspects that a radical-like intermediate compound is formed in the decarboxylation of pyruvic acid and that this intermediary is very reactive and is readily condensed. The compound cannot be acetaldehyde itself, since acetaldehyde does not tend to condense with itself to form acetoin under the conditions prevailing in animal tissues. Other aldehydes, like propanaldehyde, also react in the organism with pyruvic acid, forming an acetoin with five carbon atoms. In this condensation the second carbon of pyruvic acid yields the keto group of the acetoin, while the aldehyde yields the alcohol group (10). The disappearance of acetaldehyde in the whole animal is only interfered with if liver function is disturbed, but not if the kidneys have been extirpated (190). Brain tissue also catalyzes the formation of acetoin (271).

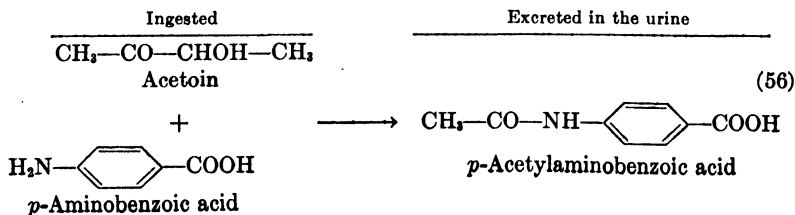


(55)



Acetoin can be reduced in the tissues, but a large percentage is oxidized as the substance passes through the body. When Westerfeld and Berg (309) fed 25 g. acetoin to a dog, they were able to recover only 4 g. of 2,3-butylene glycol and a little acetoin from the urine. So far no direct connection has been demonstrated between fat metabolism and acetoin condensation. It is probable that acetoin formation is only an alternate route in fat metabolism, since the acetaldehyde content of blood is very low, varying between 0.03 and 1 mg. per 100 g., according to Stotz (270).

Acetoin or diacetyl apparently can be reversibly split to two moles of acetic acid by oxidation, since Doisy and Westerfeld (74) observed a pronounced rise in the excretion of acetylaminobenzoic acid in the urine after feeding acetoin, butylene glycol, and *p*-aminobenzoic acid

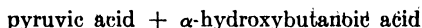
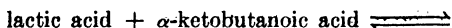


(56)

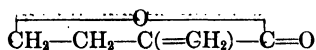
simultaneously (eq. 56). Thus formation of acetoin is not the metabolic blind alley like perhaps the formation of acetone.

This is confirmed by the findings of Green, Stumpf, and Zarudnaya (111a) of a diacetyl mutase in animal tissues, dismutating diacetyl to acetoin and acetic acid.

In the range of the lower fatty acids a large number of enzyme reactions still remain to be discovered and integrated into the over-all picture. This is demonstrated, for example, by the experiments of Friedmann and Maase (108), Weil-Malherbe (305) and Hoff-Jørgensen (128). They have shown that an α,β -dihydroxybutanoic acid, probably *d,l*-erythrodihydroxybutanoic acid, is a good hydrogen donor. Kleinzeller (149) reports that kidney sections readily oxidize butanoic acid, β -hydroxybutanoic acid, crotonic acid, vinylacetic acid, γ -hydroxybutanoic acid, and α,γ -dihydroxybutanoic acid, but not β,γ -dihydroxybutanoic acid and tetronic acid. Martius and Maué (198) have shown that one of the four stereoisomers of hydroxycitric acid is also a good hydrogen donor. Dakiñ (69) observed that hexanoic acid, β -hydroxyhexanoic acid, β -ketohydroxyhexanoic acid, and α,β -unsaturated hexanoic acid all yield only acetone in liver diffusion experiments, but no methyl propyl ketone. Actually the ketone was not isolated, but indirect analysis made it probable that acetone was really produced. Breusch and Ulusoy (49) were able to explain the mechanism of the findings of Dakiñ. According to Toropowa (292), the following equilibrium can be established with lactic dehydrogenase:



α -Methylene- γ -hydroxybutanoic acid lactone:



occurs in *Erythronium americanum*; this may be the long-sought precursor of isoprene and its polymerization products (56).

We thus see that the metabolism of the lower fatty acids permits drawing conclusions concerning that of the higher fatty acids only with caution, since lower fatty acids are also formed in the course of carbohydrate metabolism and apparently are capable of undergoing a relatively large number of catabolic and anabolic reactions which are still unknown to us.

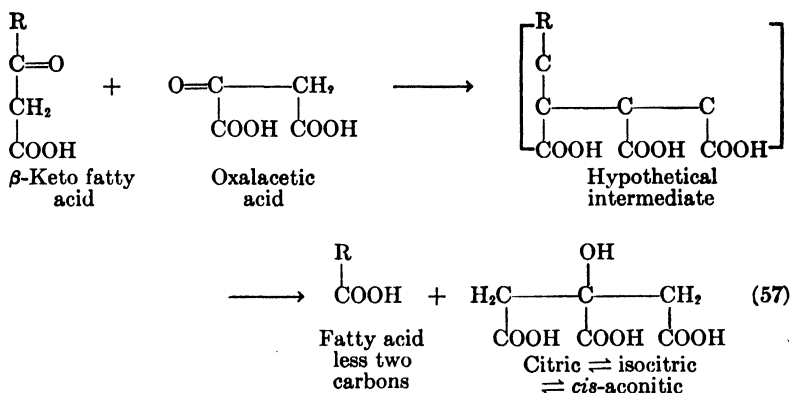
X. Further Catabolism of the Two-Carbon Fragments Resulting from the Oxidation of Fatty Acids

Simple or alternating β oxidation makes the chain of the fatty acids available in the form of two-carbon fragments. Until recently, little was known concerning the fate of these two-carbon fragments. It did not seem probable that these fragments occur as free acetic acid in any large quantities, since the acids which result from it through oxidation, such as glycolic acid, glyoxylic acid, and oxalic acid are too slowly oxidized, and since some of them, like oxalic and glyoxylic acids, are toxic in the quantities in which they would have to occur in intermediary metabolism (148). To know that acetic acid condensed to acetoacetic acid in the liver (24,84,109,193,204,205,262,279) did not help either, since the only known metabolite of acetoacetic acid, namely acetone, apparently is oxidized with difficulty in the body and should therefore be excreted mainly unchanged.

Orten and Smith (224) found in 1936 that, after they fed dicarboxylic acids containing four carbons, like *l*-malic acid, succinic acid, maleic acid, and also malonic acid, they could isolate citric acid in the urine. Simola (249) observed that feeding β -hydroxybutyric acid increased the content of α -ketoglutaric acid and of citric acid in the urine. According to Simola and Krusius (248), ingested pyruvic acid also increases the excretion of α -ketoglutaric acid in the urine. These authors assumed that β -hydroxybutanoic acid undergoes methyl oxidation to *l*-malic acid, as was already suggested previously by Kühnau (164), who unfortunately used a technique that is not clear, showing that this reaction takes place in the liver to a limited extent and represents an alternate route. Mårtensson (195) has shown that the excretion of citric acid can be better ascribed to the toxicity of high dosages of C_4 -dicarboxylic acids (as through toxic maleic acid). The mechanism of the formation of citric acid remained unexplained.

Breusch (38,39) was able to show a way in which two-carbon fragments may be oxidized. He found that acetoacetic acid, and, to a smaller extent, other β -keto fatty acids, react with oxalacetic acid in muscle to give citric acid or a C_6 -tricarboxylic acid in equilibrium with citric acid (reaction 57).

In 1943 Rosenthal and Wieland (311) independently obtained the same results with kidney, but did not recognize the fundamental role played by this condensation in the catabolism of fatty acids in the



animal organism. Breusch had already observed in 1939 (36) that only the kidneys were capable of condensing pyruvic acid with oxalacetic acid, in addition to condensing two moles of oxalacetic acid to "citric acid" (analyzed by the method of Pucher, Sherman, and Vickery, 228).

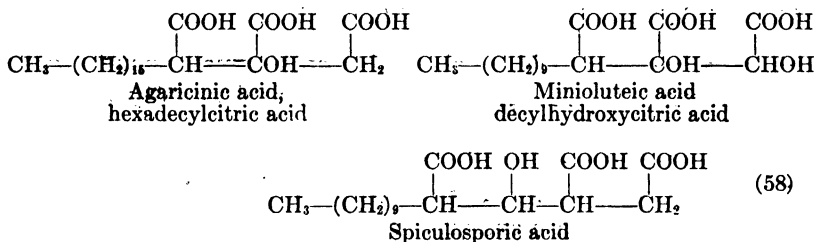
It is still undecided whether other β -keto fatty acids beside acetoacetic acid also react directly, since they too form C_6 -tricarboxylic acids with oxalacetic acid by the action of enzymes, though to a smaller extent (41). It may be that the higher β -keto fatty acids are first split to two-carbon fragments and then are condensed with oxalacetic acid either directly or after recondensation to acetoacetic acid. This question needs additional research, since the tricarboxylic acids formed, which have been analyzed as "citric acids" colorimetrically, have not yet been isolated; in contrast to the acetoacetic acid-oxalacetic acid condensation (see also 47). Hence equation 57 remains only an outline.

As yet we do not know how many intermediary steps the condensation reaction passes through and whether citric acid is formed first, or whether isocitric acid or *cis*-aconitic acid is formed first. Buchanan Sakami, Gurin, and Wilson (51,52) claim that *cis*-aconitic acid is formed as the most probable product of condensation. These three C_6 -tricarboxylic acids are in equilibrium in almost all living cells, as a result of the action of the enzyme aconitase. In an equilibrium mixture citric acid constitutes 80% and can be analyzed and isolated most rapidly (196). This equilibrium reaction was suspected by Koenigs (156) in 1892. On the basis of the almost quantitative yield

of citric acid from acetoacetic acid Wieland and Rosenthal concluded that all four carbon atoms of acetoacetic acid form citric acid.

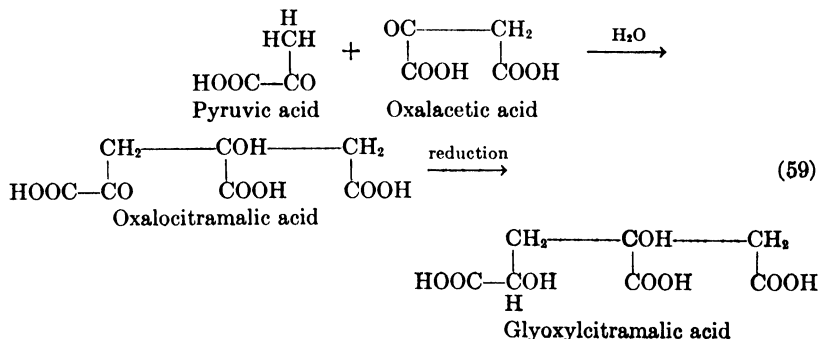
Wieland and Rosenthal (311), as well as Breusch and Keskin (41), were able to isolate the biological condensation product, or citric acid, or the quinidine salt, which melts at 128–130°C. and forms beautiful crystals. Weinhouse, Medes, and Floyd also used this second method (308). Definitive proof is only given by the isolation of citric acid, since Breusch and Tulus (47) were able to show that the colorimetric micromethod of Pucher, Sherman, and Vickery (228) is not sufficiently specific, as far as the definition of structure is concerned.

It is interesting to note that paraffin-substituted citric acids and other substances of similar structure occur in nature, just as they are postulated according to the condensation shown in equation (57). These are agaricinic acid (59,238), minioluteic acid (21), and spiculosporic acid, obtained by Clutterbuck, Ralstrick, and Rintoul (64) and caparatic acid, which Asano and Ohta isolated (7b) from lichens; the latter is an agaricinic acid the paraffin side chain of which has only fourteen rather than sixteen carbons.



Martius (196,199) in 1937 discovered that citric acid is degraded to α -ketoglutaric acid by way of isocitric acid. Neuberg and Ringer (220) and then Weil-Malherbe (304) were able to show that α -ketoglutaric acid can be transformed to succinic acid through the action of enzymes. In the last two reactions two carbon atoms are split off in the form of two molecules of carbon dioxide (see equation 60). Thus succinic acid is formed, which is then oxidized to oxalacetic acid (117,280) by way of the equilibrium fumaric acid \rightleftharpoons L-malic acid (9,78–80,200). Oxalacetic acid in turn can again react with acetoacetic acid to give a C_8 -tricarboxylic acid which then is oxidized to C_4 -dicarboxylic acids and two molecules of carbon dioxide. This then is the method by which the two-carbon fragments of the fatty acids are catabolized, that is, by constantly passing through the C_6 -

tricarboxylic acid cycle (eq. 60). Krebs and Johnson (159), following an *in vitro* proposal (154), advanced a similar theory in 1937. They postulated that pyruvic acid and oxalacetic acid condense to give a seven-carbon product through the action of enzymes; the latter product was supposed to form citric acid or one of the C_6 -tricarboxylic acids in equilibrium with it. Thomas (287), Barron (8), Breusch and Kara (40), and Stare, Lipton, and Goldinger (264) disagreed with that hypothesis. Breusch (35) was able to show that, if oxalacetic acid is merely neutralized in concentrated solution, condensation products are formed which are also analyzed as citric acid, if the colorimetric method of Pucher, Sherman, and Vickery (228) is used since that method is nonspecific (47). Even minimal concentrations of Ca will catalyze this condensation (43). The reaction product is not stable when boiled with alkali, but probably as a result of reduction to the corresponding hydroxy acid, its α -keto acid forms an alkali-stable "citric acid," which is not identical, however, with citric acid. The author assumes the reaction proceeds as in (59). Oxalocitramalic unstable to boiling alkali, and glyoxylcitramalic, stable to boiling alkali, both give the pentabromacetone reaction of citric acid.



Definitive proof can only be given by isolation of the actual condensation product. Extensive unpublished experiments conducted at the institute of the author have shown that the "citric acid" formed according to Krebs and Johnson (159) cannot be isolated as a quinidine salt by the method worked out by Breusch and Keskin (41), even though it was possible to isolate most of only 25% pure citric acid which had been added to the same tissue extract. Similarly the "citric acid" which Breusch and Tulus (44) had analyzed as a product of the condensation of oxalacetic acid with higher β -hydroxy fatty acids could not be isolated as the quinidine salt, even though in this

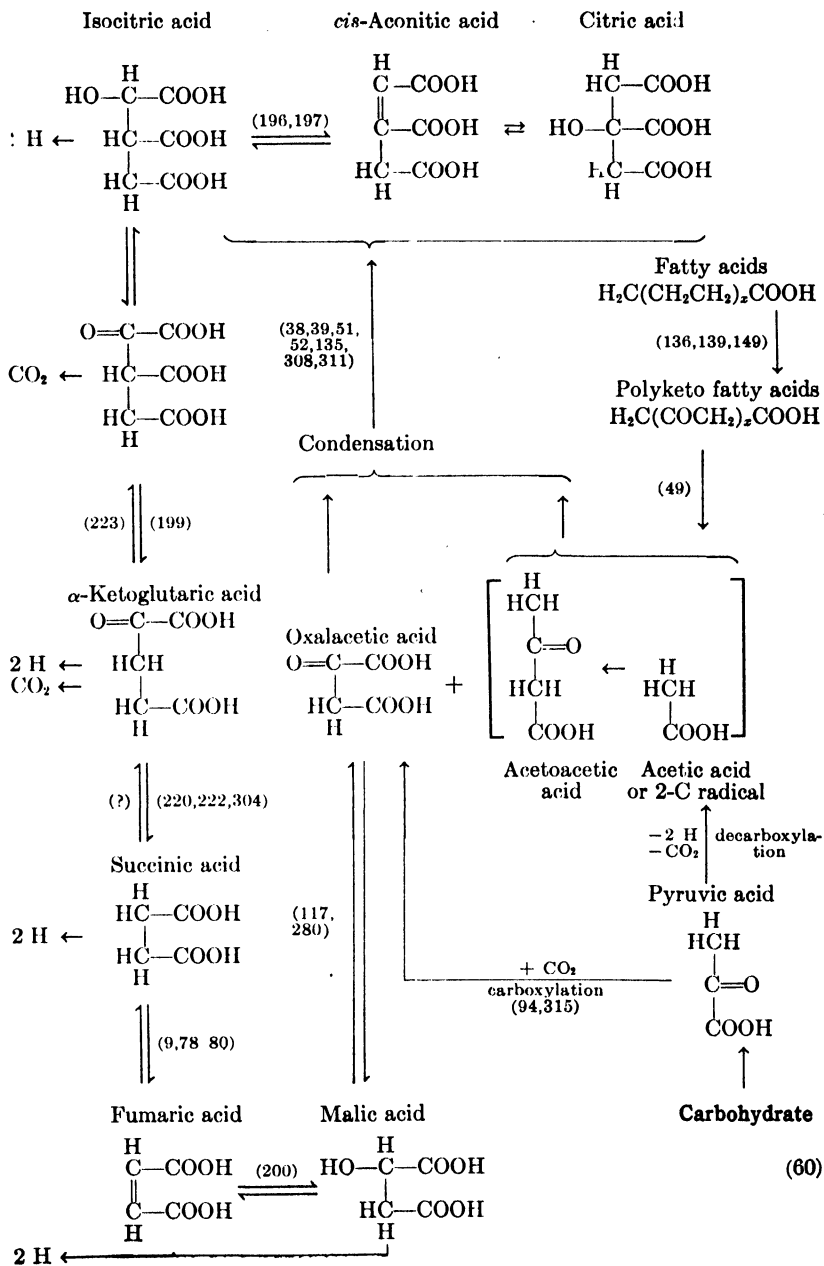
case too citric acid which had been added could be isolated. It may well be that we are dealing here with an artifact due to the effect of the higher fatty acids.

At first Krebs and Eggleston (161,162), who because of the war were working with tissues obtained at stockyards, felt unable to accept the theory postulating the condensation of acetoacetic acid and oxalacetic acid by the action of enzymes. Now, however, after the definitive experiments with the C^{18} isotope (51,52,308), they acknowledge the correctness of the theory (personal communication). The problem of which of the three C_6 -tricarboxylic acids represents the first condensation product has not yet been solved nor do we have an answer to the question of the possible formation of phosphorylated intermediary compounds. ■

It is now certain that pyruvic acid can form acetic acid in the liver by way of a reactive two-carbon fragment and then can form acetoacetic acid through condensation. We therefore can assume that pyruvic acid also takes part in the acetoacetic acid-oxalacetic acid condensation through the same mechanism. If, in spite of this, *in vitro* incubation experiments of muscle tissue with pyruvic acid and oxalacetic acid do not result in the formation of true citric acid, this may be due to the fact that large amounts of keto acids may have damaged the respiration of the muscles to such an extent that pyruvic acid no longer can be oxidized to two-carbon fragments in any sizable quantities and the artificial conditions which prevail cause an artificial condensation product to be formed under the influence of tissue calcium.

So far only one C_6 -tricarboxylic acid cycle has been demonstrated. Its essential mechanism is the condensation of acetoacetic acid (perhaps as a two-carbon, radical-like fragment) with oxalacetic acid to form one of the three C_6 -tricarboxylic acids which are in enzymic equilibrium, namely, isocitric acid \rightleftharpoons *cis*-aconitic acid \rightleftharpoons citric acid (reaction scheme 60).

The condensation of acetoacetic acid with oxalacetic acid to form citric acid (or *cis*-aconitic acid \rightleftharpoons isocitric acid) at first was rejected by Martius (197), but then was confirmed by him. Similarly Buchanan, Sakami, Gurin, and Wilson (51,52) confirmed the reaction scheme. They were working with acetoacetic acid containing C^{18} in the carboxyl group. They also were able to isolate the α -keto-glutaric acid (C_6) formed after the condensation of acetoacetic acid



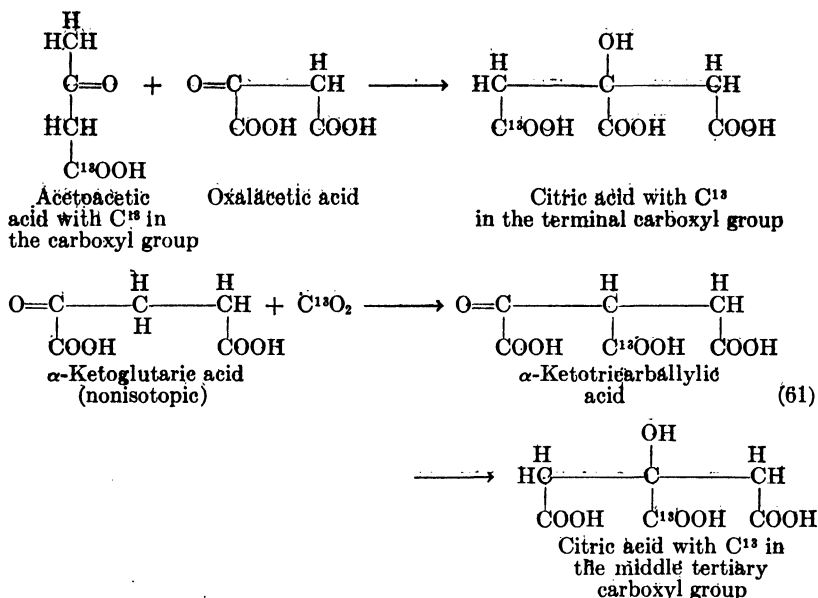
with oxalacetic acid to form a C_6 -tricarboxylic acid which in turn is oxidized to α -ketoglutaric acid. The authors concluded on the basis of the C^{13} content of the carboxyl group and of the keto group that the condensation probably had not proceeded by way of the symmetrical citric acid, but rather by way of *cis*-aconitic acid (see equation 60). It may be possible, however, that the formation of α -ketoglutaric acid proceeds by an entirely different, unknown route, since Evans and Slotin (92,93) have shown with radioactive carbon dioxide ($C^{11}O_2$) that pigeon liver is capable of synthesizing α -ketoglutaric acid from pyruvic acid and carbon dioxide.

According to Lardy and Phillips (176), this condensation also takes place in the respiration of sperm. Hunter and Leloir (135), who worked with kidney suspensions, also confirmed this condensation reaction. They found this condensation will result in maximum yields of citric acids only if ketoglutaric acid or glutamic acid is oxidized simultaneously.

The final proof of the presence of this enzyme-catalyzed condensation was furnished by Weinhouse, Medes, and Floyd (308). They found that, when they incubated acetoacetic acid containing the C^{13} isotope in the carboxyl group with oxalacetic acid in kidney tissues, the citric acid which they isolated contained C^{13} in one of the outer carboxyl groups. They report that according to the C^{13} content, 70% of the citric acid formed arose as a result of the condensation reaction described by Breusch, and Wieland and Rosenthal.

In a further, more detailed investigation (106a) Floyd, Medes, and Weinhouse, using tissue homogenates in the presence of Ba^{2+} ions, as did Wieland and Rosenthal (311), found that, under these conditions, in kidney—58%, in muscle—37%, and in brain—25%, of the isolated citrate was derived from the incubated C^{13} acetoacetic acid. The rest of the nonisotopic citric acid they suppose was derived from the condensation of oxalacetic acid with an acetic acid-like two-carbon radical, which, according to a theory of Martius (197), may be a chief intermediary metabolite of all kinds of food. The authors (106a), using C^{13} -containing bicarbonate and α -ketoglutarate, found that an appreciable part of the citric acid formed while being incubated with kidney is due to the Ochoa reaction (223), the enzymic carboxylation of α -ketoglutarate to ketotricarballylic acid yielding isocitric acid which, in turn, yields citric acid (eq. 61).

Breusch and Keskin (41) have investigated the properties of the



condensing enzyme system more closely. They designated it "citrogenase" provisionally, not knowing, however, the actual steps involved. This condensation system is particularly prevalent in muscle and kidney tissue. It does not occur so much in brain tissue, and seems almost completely absent in liver tissue, but this may only be apparent, since liver may be able to oxidize any formed citric acid particularly rapidly (45,175). Lehninger (179) was able to observe the formation of citric acid (or another C_6 -tricarboxylic acid) from acetoacetic acid and oxalacetic acid in liver after slowing the degradation of citric acid by the addition of malonate.

This enzyme system is completely lacking in the lungs and the spleen. This is in accord with the report of Snapper and Grünbaum (255,257), who found that muscle and kidney catabolize large quantities of acetoacetic acid, while liver tissue catabolizes less and the lungs and the spleen do not catabolize any. In general it can be said that the lungs, the spleen, the placenta, and the parotid glands do not react with α - or β -keto acids, or only do so very slightly (46).

The optimum pH of the condensing enzyme system is in the neighborhood of 7.5 in muscle. It is inhibited by arsenite, but not by sodium fluoride or iodoacetic acid. The system is inactivated when

heated to 60° for ten minutes. It inactivates itself in minced pigeon muscle tissue 50%, if kept at room temperature for five hours. Even minimum quantities of acetoacetic acid (three to five milligram per cent) will give quantitative reactions, while higher concentrations of oxalacetic acid are required.

According to Breusch it is possible to extract the enzyme system from tissues by means of a bicarbonate solution. Hunter and Leloir (135) showed later that the enzyme does not actually go into solution, but that the colloidal particles of the suspension which have been extracted out are the real carriers of enzyme action.

In 1913 McKenzie and Widdows (203) advanced the suggestion that acetic acid, in nascent state may occur in the form of a ketene-like radical in biochemical reactions. This suggestion was supported by Martius (197). He was able to show that, when pyruvic acid is broken up by oxidations, the intermediate two-carbon fragment may either combine with oxalacetic acid, with the formation of small quantities of "citric acid," which were not isolated, however, or with acetaldehyde, with the formation of diacetyl and acetoin. Neither free acetic acid, however, nor acetaldehyde can undergo these reactions. Lehninger (179) and Weinhouse, Medes, and Floyd (308) also believe that radical-like two-carbon fragments are the actual compounds undergoing reaction. Weinhouse, Medes, and Floyd express the belief that that substance which gives the greatest yields of citric acid with the help of enzymes, *i.e.*, acetoacetic acid, first is broken up into two-carbon fragments which in turn undergo the condensation reaction. This belief is strengthened by the fact that citric acid is quantitatively formed from all four carbon atoms if small quantities of acetoacetic acid are used (311). The original outline of the reaction proposed by Breusch (see eq. 57) indicates, however, that only half of the molecule should react. Yet, equations 62 and 64 indicate that the remaining two-carbon fragment can react with another molecule of acetic acid to form acetoacetic acid, which then can again react. Any further conclusions will have to await additional studies of the mechanism of the condensation reaction. Lipmann (187) suggests that the reactive intermediary product is an acetyl phosphate which is formed as a result of the enzymic decomposition of pyruvic acid. Experiments by Hunter and Leloir (135) indicate, however, that acetyl phosphate is not identical with the reactive two-carbon fragment. Lynen (191), working with yeast, observed

the formation of citric acid from acetic acid and oxalacetic acid, but here too previous condensation of acetic acid is not excluded. Yeast also oxidizes acetic acid directly to citric acid. Sonderhoff and Thomas (260), carrying out this reaction, obtained citric acid- d_2 from acetic acid- d_3 . This reaction does not take place in warm-blooded animals.

Acetoacetic acid, the primary metabolite of fatty acids in liver, and, to a smaller extent, higher β -keto fatty acids eventually formed in muscle tissue, undergo condensation with oxalacetic acid in muscle and kidney tissues, resulting in the formation of C_6 -tricarboxylic acids due to the action of an enzyme system (citrogenase). These acids, citric acid \rightleftharpoons cis-aconitic acid \rightleftharpoons isocitric acid, are in an enzymic equilibrium with one another, of which citric acid makes up 80%. These acids lose two carbon atoms through oxidation and then are readily oxidized to oxalacetic acid (C_4), which in turn undergoes new condensations and thus burns fatty acids at the rate of two-carbon fragments; this is called the C_6 -tricarboxylic acid cycle.

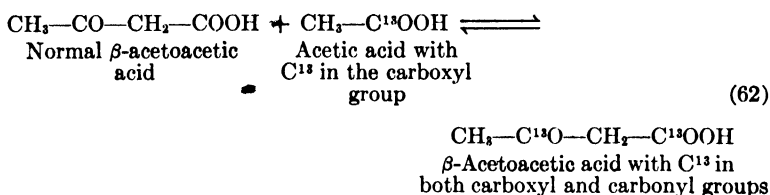
XI. Ketone Body Formation and Degradation

Embden and Loeb (84) in 1913 as well as Friedmann (109) discovered that the animal organism can convert acetic acid to acetoacetic acid. Blixenkrone-Møller (24), MacKay, Wick, and Barnum (193), and Stadie, Zapp, and Luckens (262) reported further evidence pointing in this direction. The definitive proof was furnished in 1942 by Swenseid, Barnes, Hemingway, and Nier (279), who fed rats acetic acid "tagged" with C^{13} . Medes, Weinhouse, and Floyd (204,205) obtained the same result with liver sections. The same authors (306) observed that octanoic acid "tagged" with C^{13} in the carboxyl group formed acetoacetic acid in the liver, C^{13} being found in both the carbonyl and the carboxyl groups of the acetoacetic acid molecule (equation 15, page 359). Lehninger (180), working with a liver preparation, reports that the oxidative transformation of free fatty acid to acetoacetic acid requires the presence of adenosine triphosphate and magnesium ions.

Fatty acid oxidation proceeds, in the liver at least, by way of a primary formation of acetic acid or of some other two-carbon fragment, with the formation of acetoacetic acid as the second step.

Medes, Weinhouse, and Floyd (206), who have investigated this important question in more detail, found that acetic acid is oxidized

by liver, kidneys, and heart. When liver slices are incubated with acetic acid, acetoacetic acid will be formed, while kidney slices bring about the formation of acetoacetic acid to a much smaller extent. Even so, it was possible to demonstrate with kidney slices as well that when $\text{CH}_3\text{C}^{13}\text{OOH}$ and $\text{CH}_3\text{COCH}_2\text{COOH}$ are incubated simultaneously, there is a partial formation of $\text{CH}_3\text{C}^{13}\text{OCH}_2\text{C}^{13}\text{OOH}$, thus indicating that perhaps an equilibrium exists between the formation of acetoacetic acid and the disappearance of acetic acid (302). The same result was observed when $\text{CH}_3\text{CH}_2\text{CH}_2\text{C}^{13}\text{OOH}$ was incubated, $\text{CH}_3\text{C}^{13}\text{OCH}_2\text{C}^{13}\text{OOH}$ being formed in both liver and kidneys (204).



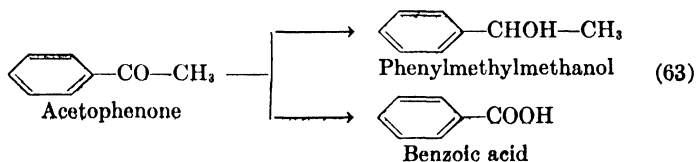
Buchanan, Sakami, and Gurin (52a) report, however, that, when liver slices are incubated with acetoacetic acid containing C^{13} in the carboxyl-carbonyl groups, no reverse splitting to two-carbon fragments takes place. In later experiments, Floyd, Medes, and Weinhouse (106a) also found that labeled acetoacetic acid, incubated with kidney homogenate, but poisoned with Ba^{2+} , did not diminish their C^{13} content, as would have been expected in the case of an equilibrium. The differences may be due to experimental conditions, but require further investigation.

Acetoacetic acid will slowly form acetone and carbon dioxide spontaneously. This is the reason that acetoacetic acid and acetone are excreted in ketonuria and diabetes, in addition to β -hydroxybutanoic acid, which is the reduction product. Creatinuria always precedes ketone body formation by a short interval (34), an observation which has as yet remained unexplained.

It appears that once acetone has been formed, it no longer can be recarboxylated to acetoacetic acid, as was shown in experiments in which acetone was incubated with C^{13}O_2 in the tissues (279). This is in contrast with the reassimilation of carbon dioxide by pyruvic acid to form oxalacetic acid, which Wood and Werkman have reported (315). Krauel and Gibson (157) report that muscle and liver tissues will not cause added acetone to disappear *in vitro*; Winkelhofer (313).

on the other hand, was able to observe a slight disappearance of acetone and of other methyl ketones from liver and other organ slices. He also observed a partial reduction to secondary alcohols, such as had been previously observed by Neuberg and Nord (219) in yeast. Krusius (163) reports that starving rats fed 8 g. acetone per kg. body weight excreted only 20% of the acetone in the urine, but excreted large quantities of β -hydroxybutanoic acid. It may be that part of the acetone disappeared through respiration in the lungs.

It is possible that some acetone is oxidized to acetic acid, as Thierfelder and Daiber (284) observed that ingested acetophenone was excreted partially reduced to a secondary alcohol, and partially oxidized to benzoic acid. Polonovski and Valdiqúé (225a) observed the formation of acetic acid from acetone in adrenal extracts. The occurrence of excess β -hydroxybutanoic acid in the urine following the ingestion of acetone can only be explained if we assume that acetone is oxidized to acetic acid, which in turn is recondensed to acetoacetic acid with subsequent reduction, unless, of course, it is liberated elsewhere as a result of the presence of acetone. The formation of aceto-



acetic acid in intermediary metabolism occurs in a clinically important disease, diabetes, which is characterized by defective sugar metabolism. According to various reports (64a, 227a, 236), diabetes is essentially a disturbance of the phosphorylation of glucose to glucose-1-phosphoric acid ester (the Cori ester). For this reason the formation of ketone bodies has long been a subject of clinical and biological investigations, yet remaining until recently one of the most obscure biochemical problems.

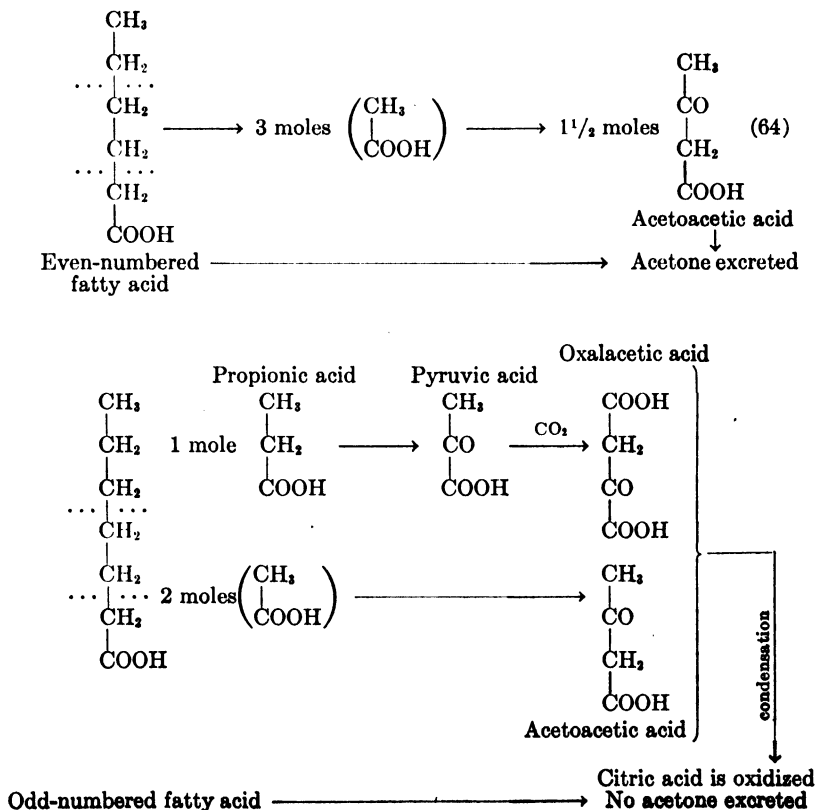
Magnus-Levy (194) suggested in 1899 that fats are the principal source of ketone bodies. Embden and Isaac (81a) and Embden and co-workers (82–85) were first to observe that, when liver is diffused with even-numbered fatty acids, ketone bodies are formed, while fatty acids with an uneven number of carbon atoms do not form them. The same thing was observed by Deuel, Hallman, Butts, and Murray (71) and by Krainick (156a). These experiments contrast with ob-

servations of Jowett and Quastel (138) and Edson (75), who found that fatty acids with an odd number of carbon atoms, such as valeric acid, heptanoic acid, and nonanoic acid, also produce acetoacetic acid, that is, they split off carbon dioxide and form acetone; this is not true, however, of propanoic acid. Investigations of MacKay, Wick, and Barnum (193) support the findings. They fed fatty acids with an odd carbon number to starving rabbits and observed the formation of ketone bodies. They suggest that the contradictory results obtained by Deuel *et al.* were due to the fact that the doses of fatty acids fed by these authors were too small. Leloir and Muñoz (181) were able to explain these discrepancies. They claim that, when they incubated liver sections, acetone was formed, but that each of the following acids incurred losses as follows: formic acid, 1.5 parts; acetic acid, 5 parts; propanoic acid, 2.0 parts; butanoic acid, 9 parts; pentanoic acid, 2.1 parts; hexanoic acid, 6 parts; heptanoic acid, 3 parts; and octanoic acid, 6 parts. Odd-numbered fatty acids may also form acetoacetic acid, but to a lesser extent than even-numbered ones. Since both odd-numbered and even-numbered fatty acids can undergo either simple or alternating β oxidation, as has been shown by the experiments of Knoop, Verkade, Flaschenträger and Bernhard, it is difficult to understand why the two-carbon fragments from odd-numbered fatty acids which result from β -oxidation cannot just as well condense to acetoacetic acid in the liver as those derived from even-numbered fatty acids.

All work on ketonuria has been carried out on animals fed a diet low in carbohydrates since the addition of carbohydrates will suppress ketonuria. This may be due to the fact that glucose will lead to the formation of oxalacetic acid, which will condense with acetoacetic acid, forming citric acid or one of the C_6 -tricarboxylic acids in equilibrium with it, and thus will result in the elimination and oxidation of acetoacetic acid.

When fatty acids with an odd number of carbon atoms in the molecule are broken down by β oxidation (scheme 64), propanoic acid is formed automatically as the last fragment; propanoic acid then is converted to pyruvic acid, or glucose, by the only α oxidation known to occur in tissues (29,114,237). Pyruvic acid is known to be able to add carbon dioxide in the liver with the formation of oxalacetic acid (94,95,316) and thus may act as an antiketogen, in accordance with Breusch's theory. The fact that there is heavy ketone formation

with even-numbered fatty acids and little ketone formation if the fatty acids have an odd number of carbons can be explained by the experimental conditions themselves. The formation of ketones from even-numbered fatty acids has nothing to do with the catabolic mechanism of either even-numbered or odd-numbered fatty acids, for, after all, no known experimental data in the biochemistry of the catabolism of fatty acids would tend to support such differentiation, particularly since the difference occurs solely when the diet consists only of fats, and not of a mixture of carbohydrates and fats. It should be recalled that during the war the population in Germany ate thousands of tons of mixtures of fatty acids which had been obtained from the oxidation of paraffins and which consisted of equal mixtures of even-numbered and odd-numbered fatty acids; yet this



did not result in any measurable changes in their metabolism (see 235 for a discussion of the composition and 158 for a discussion of the biochemistry of these fats).

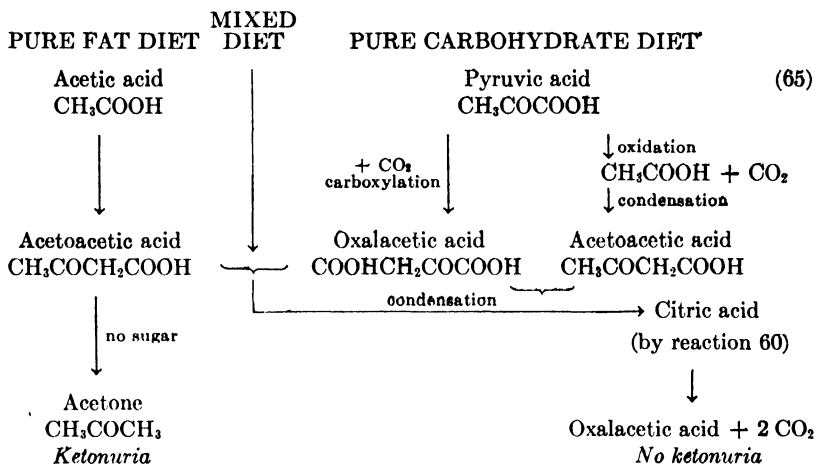
According to experiments by Appel, Böhm, and Keil (7), the ingestion of large quantities of fatty acids with an odd number of carbon atoms will result in the accumulation of depot fat which contains up to 30% fat with an odd number of carbon atoms without causing any disturbance in metabolism. Keil, Appel, and Berger (145) synthetically lengthened the chains of a mixture of even-numbered fatty acids derived from coconuts by one carbon. They thus produced odd-numbered fatty acids, and from these were made triglycerides. These two types of fats were fed to two groups of rats; it was found that the respiratory quotients, the iodine numbers of the depot fats, and the carbon to nitrogen ratios in the urine were identical in both experimental groups. Emmerich and Nebe (86) of the Thomas Institute confirmed this work. All triglycerides, regardless whether they have an even or an odd number of carbon atoms in the chain, behave identically in the presence of pancreatic and serum lipases according to Kabelitz (141).

It should be pointed out in this connection that some of the paraffins produced synthetically contain about 20 per cent of branched fatty acids which, in view of present-day knowledge of the tuberculin fatty acids, can no longer be considered harmless. Such branched chains are found in paraffins produced from carbon monoxide and hydrogen according to the Fischer-Tropsch-Gatsch process, but not in hydrogenated paraffins derived from lignite. Flössner (106) claims the former type of fat is harmless. Thomas and Weitzel (288) contradict him, pointing out that branched fatty acids can undergo ω oxidation particularly easily (see equation 41, page 378) and thus may lead to diaceturia, and hence may not be harmless for the kidneys in the long run. When synthetic fatty acids derived from lignite paraffins are administered, no diaceturia occurs, since they contain no branched fatty acids. Thomas and Weitzel also point out that the synthetic glycerol required in the artificial esterification of the fatty acids synthesized from paraffin also contains toxic constituents which can only be removed by repeated purification and which must be removed if synthetic fats are to become a suitable food for general consumption (see also reference 7).

It is quite probable that some pyruvic acid too is catabolized by

way of the condensation reaction of acetoacetic acid and oxalacetic acid to citric acid (or *cis*-aconitic acid \rightleftharpoons isocitric acid), since pyruvic acid is changed to acetic acid by the animal as shown by Gurin, Del-luva, and Wilson (116a).

Although acetoacetic acid is formed in the liver from pyruvic acid, *i.e.*, carbohydrate, by way of acetic acid and a recondensation, no ketone formation takes place in the animal, since oxalacetic acid is formed from pyruvic acid at the same time. Pyruvic acid adds carbon dioxide, according to Wood and others (94,315-317), and



acetoacetic acid thus is eliminated immediately. If there is a pronounced excess of pyruvic acid, the formation of acetoacetic acid appears to proceed more rapidly than carboxylation to oxalacetic acid, with the result that ketone bodies are formed (111), particularly if an excess of ammonium ions is present (5). These observations require further clarification. Brentano's (34) observation that every ketonuria is preceded by creatinuria should be called to mind in this connection.

We thus see that oxalacetic acid has a key position in the catabolism of fatty acids and probably also in that of carbohydrates. Unless other, yet unknown, enzyme systems are involved in the elimination of the two-carbon fragments resulting from the degradation of the fatty acid molecule, oxalacetic acid is absolutely essential for the removal of acetoacetic acid, the unavoidable intermediary product of

the liver. No other means is available to the animal organism for oxidizing acetoacetic acid in larger quantities. If oxalacetic acid or pyruvic acid, its precursor, is absent, or if an insufficient amount of oxalacetic acid is synthesized from pyruvic acid, acetoacetic acid and acetone have to be excreted in the urine.

Thus only those substances have antiketogenic action which can provide oxalacetic acid to the healthy animal. These are: glucose-pyruvic acid, lactic acid, alanine, but *not* acetic acid, ethanol, acetaldehyde, and glycine (246).

We therefore see that Rosenfeld's old slogan (241) that "the fats burn in the fire of the carbohydrates" is partly justified; they burn under the catalytic influence of oxalacetic acid, which is formed from glucose by way of pyruvic acid.

Oxalacetic acid and γ -malic acid suppress β -keto acid formation, as was shown in liver incubation experiments (81), while fructose increases the rate of elimination of acetoacetic acid from the liver (76). The addition of lactic acid and of glucose increases the rate of acetoacetic acid catabolism in the kidneys by 15 to 30% (137). Liver tissue containing little glycogen will form many ketones from fatty acids, while liver tissue containing much glycogen will form very few ketones (24). Suomalainen and Kinnunen (277) report that the addition of C_4 -dicarboxylic acids reduces the *in vitro* acetylation of amines.

The formation and excretion of ketone bodies thus is not based on any basic metabolic changes, but more on a shift of enzyme capacities. According to Blixenkrone-Møller (23) even diabetic, pancreatectomized cats oxidize 90% of about 5 g. of ketone bodies formed daily in the liver and excrete only 400 mg.

Because of its role in the clinically important disease diabetes, too much emphasis has been placed on ketone body formation. In his review of 1937 Sinclair (250) questions the value of considering the formation of ketone bodies as a measure of biochemical reactions. He feels that too many variable factors in perhaps ten to fifteen different enzymes are involved in the formation of ketones, precluding the possibility of calculating the temporary effect due to any substance which might have been fed or administered.

It is shown that the formation of ketone bodies from even-numbered fatty acids in starving animals is probably due to the inadequate formation of oxalacetic acid which is required for the oxidation of ketone

bodies by way of the C_6 -tricarboxylic acid cycle. Oxalacetic acid is formed from pyruvic acid through carboxylation, pyruvic acid being derived from glucose. An animal on a normal nutritional level will exhibit no differences in its metabolism of even- and odd-numbered fatty acids. In the starving animal, odd-numbered fatty acids result in almost no ketone formation, in contrast with even-numbered acids, since their last fragment of oxidation is a three-carbon chain which forms pyruvic acid. The latter in turn adds carbon dioxide and forms oxalacetic acid, which prevents the formation of ketone bodies.

XII. Function and Interaction of Various Organs Involved in Fatty Acid Catabolism

The various organs of the body of warm-blooded animals have chemically different mechanisms of the catabolism of fatty acids. These mechanisms are complementary in nature.

The liver, being the principal organ of metabolism, apparently has to initiate the degradation of fatty acids. The rate of the metabolism of fats in the liver is particularly high. Thus, according to Hahn and Hevesy (118), administered phosphate containing radioactive phosphorus is incorporated into phosphatides particularly rapidly in the liver and in the intestinal mucosa (7a); the same is reported by Sperry, Waelsch, and Stoyanoff (261) for fatty acids containing deuterium. Similar experiments carried on with the metabolism of fat in the brain have shown it to be particularly low. The lungs too can, for all practical purposes, be said to be inactive in the metabolism of fat (245a and 269). The half-life of deuterium-containing fat fed by Stetten and Grail (267) was only 2.6 to 2.8 days in the liver, or shorter than in other organs or in depot fat (5-6 days). Nevertheless, deuterium-containing fat which has been ingested makes its first appearance in the fat depots and only later enters the metabolic process as a whole (244). Liver phosphatides have a higher deuterium content than triglycerides (77). At starvation levels the lower fatty acids (lauric acid) of the depot fat are broken down somewhat more rapidly than the higher ones (189).

The cells of the liver, rich in lipides, are able to convert the fatty acids to water-soluble two-carbon fragments. The liver accomplishes degradation of fatty acids mainly by alternating β oxidation (equation 14, page 358). The two-carbon fragments in turn are converted to acetoacetic acid and β -hydroxybutanoic acid. No other

organ is capable of bringing about alternating β oxidation (49). No information is available as yet concerning how far phosphorylations are involved in this process.

The intermediate step of fatty acid catabolism is not through fatty acids of medium chain length. This opinion is in accordance with experiments of Appel, Böhm, Keil, and Schiller (7). Upon feeding a mixture of 50% odd- and 50% even-numbered fatty acids having a chain length of C_9 to C_{22} , these investigators found, in the milk and in the body fat, 25% odd-numbered fatty acids of chain length C_{11} to C_{19} , whereas low fatty acids were composed exclusively of C_6 and C_8 and contained no C_7 or C_9 .

The liver has only a limited capacity for further reducing acetoacetic acid to β -hydroxybutanoic acid (110, also the diffusion experiments of Snapper and Grünbaum, 255,257; of Chaikoff and Soskin, 58; Himwich, Goldfarb, and Weller, 126a). Acetoacetic acid and β -hydroxybutanoic acid, the end products of fatty acid catabolism in the liver, have a high energy content and are readily soluble in water, and thus can enter the circulation. According to Crandall, Ivy, and Ehni (65a), the blood concentration of ketone bodies is 0.5 to 1.4 milligram per cent; according to Agrell (1a), 2.8 to 3.2 milligram per cent, and according to Krautwald (158a), 0.35 to 3.5 milligram per cent. In hunger or after fat ingestion, the concentration increases to 8 milligram per cent. A hungry dog forms daily 21–34 g. ketone bodies, of which only 1% is excreted in the urine. These intermediary products then are completely oxidized to carbon dioxide and water in those peripheral organs which use up most of the energy, that is, the muscles (mechanical work) and the kidneys (work of concentration and resorption). This final oxidation proceeds by the tricarboxylic acid cycle described by Breusch and Wieland and Rosenthal (equation 60, page 397). Only muscle and kidney tissues catabolize acetoacetic acid in any large quantities (23,70,77,115,255,257). This agrees with the findings of Breusch and Keskin (41), who found that muscle and kidney contained large quantities of the enzyme system called "citrogenase," while the liver contains only small quantities. This is the enzyme system which catalyzes the condensation of oxalacetic acid with acetoacetic acid to form one of the three C_6 -tricarboxylic acids, *i.e.*, citric acid, *cis*-aconitic acid, and isocitric acid.

There can be no doubt that muscle and kidney, and possibly even liver, are capable of also directly catabolizing fatty acids, but their

capacity to do so is very small. Cuthbertson (66a) could not find a measurable decrease of fats in working muscles (115). Since both muscle and kidney lack the enzyme system necessary for alternating β oxidation, *i.e.*, the system (49) necessary for splitting the β,δ,ϵ -polyketo fatty acids (equation 14, page 358), we must assume that whatever small amount of breakdown of fatty acids does occur there, takes place by the classical route of β oxidation. Here the first two carbon atoms of the β -keto acids which are formed are removed before the chain undergoes further oxidation. The existence of different chemical mechanisms in the various organs has been shown by experiments of Snapper and Grünbaum (258). The oxidation may take place either through direct condensation of the β -keto fatty acids with oxalacetic acid to form a C_6 -tricarboxylic acid, in accordance with the reaction scheme shown in equation 57 (page 393) which involves a shortening of the chain by two carbon atoms, or through hydrolytic splitting of the first two carbon atoms from the β -keto acid with subsequent recondensation of two two-carbon fragments; these would form acetoacetic acid, which in turn would condense with oxalacetic acid to form a C_6 -tricarboxylic acid, in accordance with equation 60. It is undecided so far which of the two routes is actually taken.

According to Breusch and Ulusoy (49), the greatest capacity of formation of ketone bodies (acetoacetic acid and β -hydroxybutyric acid) by the liver in alternately splitting oxidized polyketo fatty acids is about 150 g. per kg. per day. For an adult man of 75-kg. body weight with a 2-kg. liver this is about 300 g. per day. If one assumes a ketone body content of the arterial blood of 1.5 milligram per cent, or, after fat ingestion, 8 milligram per cent (1a,65a,158a), and further assumes that in both cases the venous blood contains 1 milligram per cent, then, for every liter of blood perfused through muscles and kidneys, 5 mg. of ketone bodies disappear normally, and, after fat ingestion, 70 mg. of ketone bodies disappear. According to Rein (235a), in an adult of sedentary occupation, about 6000 liters of blood pass daily through the heart, of which 70% (4200 liters) passes through muscles and kidneys. With the passage of this 4200 liters of blood, 21 g. of ketone bodies disappear in a fasting body, or, after fat ingestion, a maximum of about 300 g. of ketone bodies disappears.

This is in good agreement with the above-mentioned maximum capacity of the liver to produce ketone bodies, namely, about 300 g. per day.

300 g. of ketone bodies, considered as acetoacetic acid, is derived from about 200 g. of fat. This amount also agrees closely with the maximum fat-resorption capacity of a normal adult man—being about 300 g. per day according to Rubner (242a) and Arnschink (7c). Of the maximum quantity of resorbed fat, a part is first deposited visibly, so that the actual combustion capacity is lower. This is also in accordance with the clinical data derived from severe diabetes, in which the maximum amount of ketone bodies excreted in the urine is about 120 g. daily. This 120 g. is not all the liver produces as, even in severe diabetes, part of the ketone bodies are metabolized (283a). Therefore, the actual maximum production capacity of the liver is higher. To this, the amounts of citric acid are to be added which are already synthesized in the liver from acetoacetic acid and are either burned in the liver or transferred to the blood for peripheral combustion.

All these estimates are based on preliminary data obtained under different conditions. Other authors have also often dealt in their papers with ketone bodies, but without explaining whether they analyzed for acetone as derived from acetoacetic acid alone or if they included analyses of β -hydroxybutyric acid.

It is concluded that the greatest part of the catabolism of fatty acids in warm-blooded animals is brought about by a gradual interaction of the muscles and kidneys, on the one hand, and the liver on the other. It is accomplished in such a way that the liver produces acetoacetic acid and β -hydroxybutanoic acid from fatty acids by alternating β oxidation, by way of two-carbon fragments. These acids then are completely oxidized in the muscles and the kidneys by way of the C_4 -tricarboxylic acid cycle. In addition, some simple β oxidation probably also takes place in muscle and kidney along with some formation of citric acid in the liver for direct or peripheral combustion.

XIII. Common Terminal Oxidation of Fatty Acids, Carbohydrates, and Amino Acids

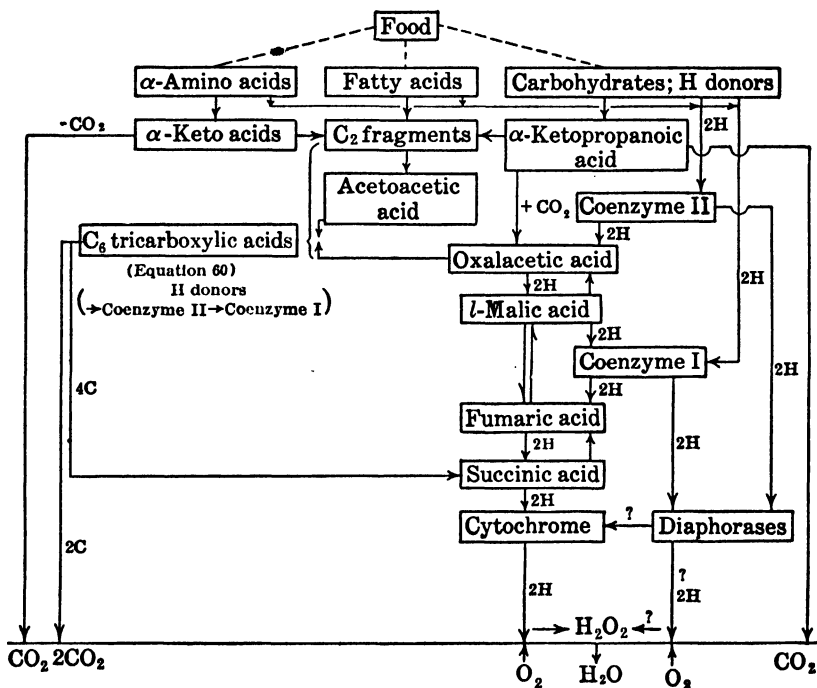
Oxalacetic acid represents a common point of the catabolic mechanisms of fatty acids and carbohydrates, for oxalacetic acid also is an intermediate in carbohydrate metabolism, acting as hydrogen transporter between the carbohydrates and oxygen. Working with this theory of the hydrogen transporter role of the C_4 -dicarboxylic acids Szent-Györgyi and co-workers (280) and later Breusch (37) were able

to produce much material that was experimentally well founded. Later this work was largely forgotten, the flavin enzymes (diaphorase 1 and 2) having been discovered which transfer hydrogen directly from the coenzymes to oxygen. The reduction of carbohydrates by oxalacetic acid, *i.e.*, the removal of hydrogen from them (probably by way of coenzyme II) with the formation of *l*-malic acid is one of the most powerful reactions, from a conversion rate point of view, that occur in the living organism. (According to Breusch (ref. 37), the reducing capacity is 2.4 g. oxalacetic acid per 100 g. pigeon muscle per hour.) It seems improbable that such an important chemical cellular process has no chief function in the main metabolism of the cell, the transport of hydrogen, except among the diaphorases. The reason that the significance of the role of oxalacetic acid in the transport of hydrogen may have been overlooked by most investigators is that the enzymes involved lose half of their activity within thirty minutes after the death of the experimental animal. As a result, most, or all, of the activity of this enzyme important in tissue metabolism will have been lost, if the usual method of the preparation of tissue sections is employed. Furthermore, rats are used as experimental animals in most laboratories. They are unsuited for a study of this reaction, however, since the available quantities of rat tissue needed for a polarimetric investigation of the formed *l*-malic acid are too small. Pigeon muscle and organs of cats which may yield 40–60 g. for each experiment are better suited to this purpose.

If the hypothesis with regard to the role of the C_4 -dicarboxylic acids and particularly that of oxalacetic acid in the metabolism of carbohydrates proves correct, oxalacetic acid is the principal substance required for the oxidation of both sugars and fats. This then permits setting up a scheme of reactions for all of the food constituents, *i.e.*, fatty acids, carbohydrates, and proteins (37). Every one of these reactions, with the exception of the position of coenzyme II, has been proved qualitatively. Their quantitative interaction, however, still awaits thorough investigation.

The reaction scheme seems to be more complete, since the probable route of insulin action in muscle seems to have been established (64a, 109a, 227a, 236) as the transformation of glucose of blood to glucose phosphates in muscle. This reaction is necessary for the formation of pyruvic acid from glucose, and oxalacetic acid seems to be synthesized chiefly from pyruvic acid by CO_2 addition according to the

Wood-Werkman reaction. Bueding, Fazekas, Herrlich, and Himwich (52b) observed that the blood level of pyruvic acid in dogs without a pancreas is not raised by glucose ingestion as normally occurs; it is only after injection of insulin that this blood level of pyruvic acid—necessary for fatty acid combustion with the help of oxalacetic acid—increases. According to Brentano (34), it is insulin that effectively prevents the formation of ketone bodies, while adrenaline prevents the elimination of ketone bodies from the blood. Houssay (131a), by his discovery of a diabetogenic hormone in the hypophysis, has shown that hormonal regulations are superimposed on the biochemical reactions catalyzed by insulin.



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LIPOXIDASE AND THE AUTOXIDATION OF UNSATURATED FATTY ACIDS

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I. Introduction

Autoxidation of the unsaturated fatty acids has long been subject to much investigation, stimulated a great deal by the technical importance of this process for the "drying" of paints and varnishes and in the development of rancidity in food products containing unsaturated fatty acids.

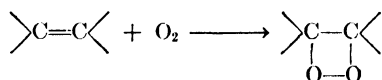
* National Research Council Fellow in Physiological Chemistry, 1946-47, at the Medical Nobel Institute, Stockholm, Sweden.

The field has gained considerable interest from a biochemical point of view since the discovery of a special enzyme, lipoxidase, which catalyzes the peroxidation of certain unsaturated fatty acids with molecular oxygen. The enzyme acts only upon the fatty acids containing methylene-interrupted double bonds, *i.e.*, primarily linoleic, linolenic, and arachidonic acid, forming apparently the same peroxidic products as those formed in the ordinary autoxidation of these acids. It might be stressed that the action of this enzyme is restricted to the acids known to be essential metabolites for a number of animals (23,25).

However, since the enzymic oxidation with lipoxidase goes only as far as the formation of the primary peroxides, mainly that phase of the ordinary autoxidation will be considered in this review. The influence of various factors (catalysts, temperature) on the later stages of the autoxidation concerned more with the polymerization or "drying" process therefore will not be treated fully in this discussion. Also, the extensive work on the action of various antioxidants, of more technological than biochemical interest, will not be considered.

II. Autoxidation of Unsaturated Compounds

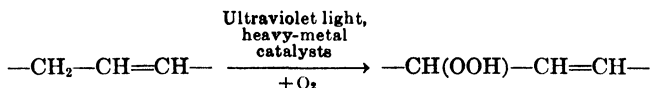
It has long been known that the unsaturation decreased during the autoxidation of unsaturated compounds. This was taken as an indication that the primary reaction was an addition of an oxygen molecule to a double bond forming a cyclic peroxide, as proposed by Engler and Bach during the last century:



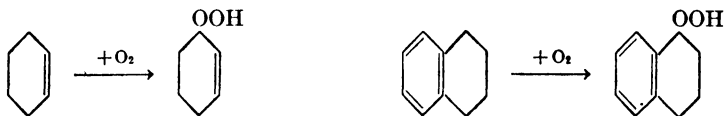
These views seem to have been generally accepted also for the autoxidation of the unsaturated fatty acids since the work of Fahrion (36,37) in the beginning of this century, although a compound with the structure shown in the above equation has never been isolated or conclusively shown to occur. Most of the earlier work (see summaries 61 and 138) was done with linseed oil or similar inhomogeneous natural products. Furthermore, the autoxidation was generally allowed to proceed very far (> one mole oxygen per double bond) or at such a high temperature that nonperoxidic decomposition products predominated, rendering any conclusions as to the structure of the

primary products very uncertain. Some recent authors (48,49,52) working with unsaturated fatty acids have supported the theory that the main primary product is a four-membered cyclic peroxide (see page 430).

In the meantime, investigations of the autoxidation of numerous cyclic compounds with isolated double bonds have led to other results. In a great many cases the primary product has been isolated in pure form and has always been found to be a hydroperoxide with the peroxidic group in alpha position to the double bond. At moderate temperatures ($< 100^\circ$) or under illumination with ultraviolet light the product is formed according to the following scheme:

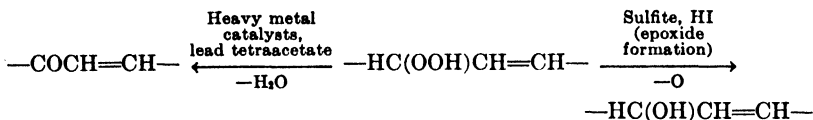


Some examples of such hydroperoxides that have been isolated are:



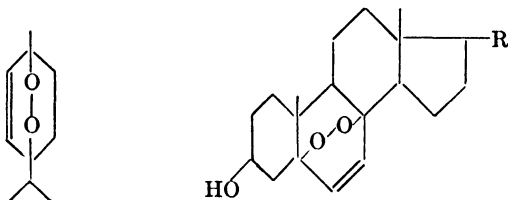
Progress in this field is to a great extent due to work of Rieche (102,103), Criegee (29,30), Farmer (43,44,47), Hellberger (63), and Hock (66-74). The reactivity of the α -methylenic group and the autoxidation of these compounds have been reviewed by Farmer *et al.* (38-41,43), Gee (51), and Waters (142,144,145).

The unsaturated hydroperoxides vary greatly in their stability but generally decompose rapidly at higher temperatures (above 100°), yielding a complicated mixture of decomposition and polymerization products. Reduction with such agents as hydroiodic acid, sulfite or aluminum amalgam in acetic acid often gives high yields of the corresponding α,β -unsaturated alcohol. Heavy metal catalysts, *i.e.*, ferrous, copper, cobalt, and manganese salts, various phthalocyanines, hemin, etc., which speed up autoxidation and formation of these hydroperoxides, also catalyze their decomposition. The predominant reaction product is then generally the α,β -unsaturated ketone. This is also the case when the hydroperoxides are treated with lead tetraacetate (33) or left in contact with anhydrous aluminum oxide.



This explains the old observations that α,β -unsaturated ketones and alcohols are sometimes formed in such good yields, when unsaturated compounds autoxidize, that the reaction can be used for preparative purposes (10,107,146). In the decompositions, especially at high temperatures, disappearance of double bonds is considerable, due in varying degrees to epoxide or glycol formation (pp. 429,433), to chain splitting at the double bond with the production of carbonyl or carboxyl groups, or to polymerization. Since these reactions proceed with different speeds at different temperatures, or with traces of different catalysts, the difficulty of clarifying the autoxidation process is easily visualized.

The autoxidation of cyclic or aliphatic compounds containing *conjugated* double bonds seems to be of a different nature. It has been postulated that these peroxides are formed by 1:4 addition to the system of conjugated double bonds forming six-membered rings. Simultaneously with the formation of these peroxides there generally occurs a very extensive polymerization unless special precautions are taken (108). Examples of monomeric peroxides of this type are ascaridol and ergosterol peroxide:



For discussions of the autoxidation of the fatty acids with conjugated double bonds, which are used extensively in paints and varnishes because of their good drying properties, the reader is referred to papers by Farmer (39,40), Morrell (99), Waters (144,145), and Heinänen (62).

III. Autoxidation of Unsaturated Fatty Acids

A. MONOETHENOID

The autoxidation of the monoethenoid oleic acid is so slow at room temperature that in order to get reasonable reaction times the oxygen

uptake has to be speeded up with heavy metal catalysts, ultraviolet light, or by using higher temperatures. The course of the oxygen uptake of the common unsaturated fatty acid esters at 37°C. is shown in Figure 1 (80).

The products formed in the autoxidation of oleic acid at 100–120° were investigated by Skellon (109), who isolated from the oxidized oleic acid two 9,10-dihydroxystearic acids, a monohydroxystearic

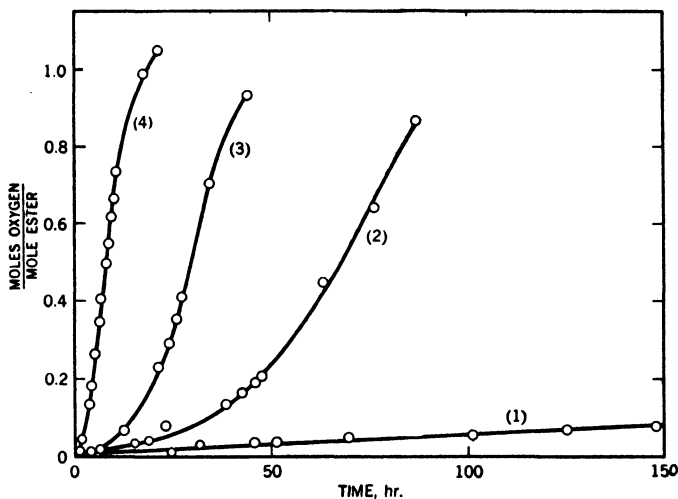


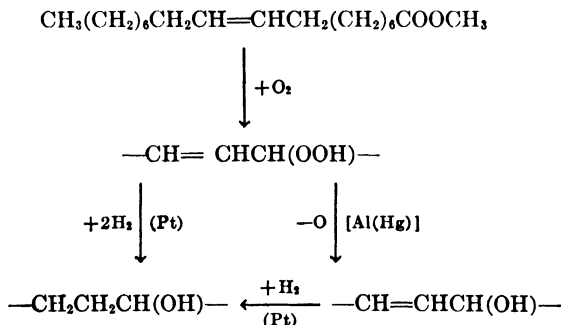
Fig. 1. Autoxidation of fatty esters in air at 37° C. (1) Ethyl oleate. (2) Ethyl linoleate. (3) Ethyl linolenate. (4) Methyl arachidonate.

acid, and a number of compounds formed by splitting the carbon chain. In a similar investigation Ellis (34,35) used cobaltous elaidate as catalyst and let the oxidations of oleic or elaidic acid proceed at 55–80° until 1–3 moles of oxygen per mole of fatty acid had been absorbed. The products contained only 1–2% fatty acid peroxide. He isolated up to 20% oxidostearic acid but noticed that the yield of epoxide was very low when no catalyst was used. Carbon dioxide, water, and oxalic, azelaic, suberic, octanoic, and nonanoic acids were also identified among the reaction products. Similar results were obtained by Deatherage and Mattill (32) and by Swern *et al.* (130). The latter used molecular distillation to fractionate the products formed from methyl oleate oxidized at 65° until the iodine number

became constant. They demonstrated that a very complex mixture of high-boiling polymerization products is formed at this temperature, with fractions with molecular weights of 1700; they also obtained evidence that a certain amount of double-bond shift had occurred.

Franke and Jerchel (48) also used a cobaltous catalyst but conducted the oxidation of methyl oleate at 37°. Even at this temperature less than half the absorbed oxygen was present in peroxidic form after an uptake of 0.2 mole oxygen per mole ester. They claimed that hydrogenation of the peroxide yielded an almost theoretical yield of α -glycolic groups, as might have been expected if a four-membered cyclic peroxide was the main reaction product. However, they have since then modified their original claim (49).

Farmer and Sutton (45) used ultraviolet light to speed up the slow autoxidation of methyl oleate in oxygen and obtained an oxygen uptake at 35° of 0.1 mole oxygen per mole oleate in five hours. All oxygen was then present in peroxidic form. By continuous molecular distillation at 65–91° they succeeded in isolating substantially pure methylmonohydroperoxido oleate, in which the presence of the double bond could still be demonstrated. On catalytic hydrogenation the hydroperoxide absorbed two moles of hydrogen and yielded a mixture of monohydroxy stearates, whereas reduction with aluminum amalgam or hydroiodic acid (131) produced a mixture of unsaturated hydroxy stearates. Catalytic hydrogenation of this product yielded the mixture of hydroxystearate mentioned earlier:

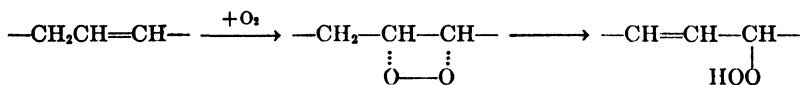


This work has been confirmed by Swift, Dollear, and O'Connor (131), who described the preparation of 90% pure methylhydroperoxido oleate by low-temperature crystallization of the reaction products.

However, in the case of oleate the location of the hydroperoxide groups has not yet been experimentally ascertained, nor have the hydroxystearic acids formed by hydrogenation been identified. The observations that the oxidation of oleate (1) is autocatalytic, (2) is stimulated by illumination with ultraviolet light, and (3) is catalyzed by the addition of substances such as benzoyl peroxide which decompose yielding free radicals are in harmony with the free-radical mechanism theory. In this connection it should be mentioned that Gunstone and Hilditch (54,65) observed that oleate oxidation is stimulated by the presence of small amounts of linoleate.

The autooxidation of methyl oleate at temperatures between 20 and 120°C. has been investigated by Atherton and Hilditch (2) and Gunstone and Hilditch (53). The former used disruptive oxidation with potassium permanganate in acetone to locate the added oxygen. With pure oleate this method gives an almost quantitative yield of azelaic and nonanoic acids. Oxidation of the product obtained after autooxidation at 20° for 34 days yielded a mixture of azelaic, suberic, octanoic, and nonanoic acid, as would be expected if a hydroperoxide formation at C-8 and C-11 had taken place. Disruptive oxidation of the product obtained after autooxidation for six hours at 120° yielded proportionately less suberic and octanoic than azelaic and nonanoic acid, indicating that another mechanism with a direct attack of the oxygen at the double bond might predominate at the higher temperature. A similar indication is also given by the abrupt increase in the reaction rate of the autooxidation near 80° (45). The iodine number after the oxidation at 120° was very low. However, as the oxygen uptake was not measured in these investigations it is difficult to estimate how much of the primary peroxides had decomposed (compare Swern *et al.*, 130).

Hilditch *et al.* (54,65) (see pp. 433,437) proposed that the isolated double bond might be oxidized by primary direct addition of oxygen to the double bond followed by a rearrangement to an unsaturated hydroperoxide:

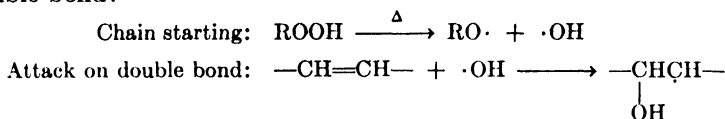


A similar rearrangement of the epoxide ring has been observed (page 434). In the case of oleic acid this mechanism should yield only the

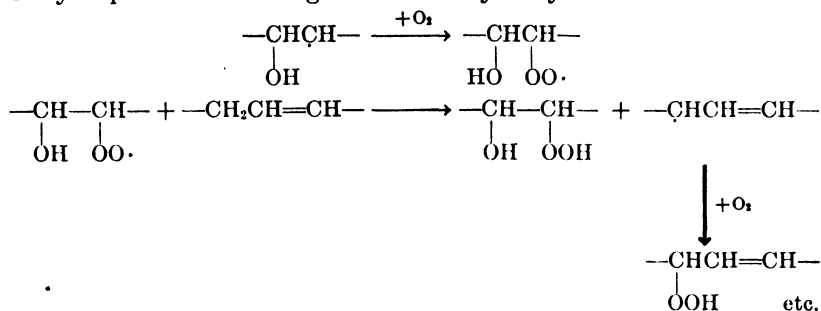
9- and 10-hydroperoxides but no 8- or 11- as postulated by Farmer *et al.*

In connection with the mechanism proposed by Hilditch it should be pointed out that in many instances the oxygen appears only in the alpha position to the original double bond (see cholesterol, etc.). However, Farmer *et al.* have discussed the possibility that the chain-initiating radicals might be formed by the addition of oxygen to a double bond (see page 437) and that these radicals could then start the chain reaction involving α -methylene hydrogen atoms.

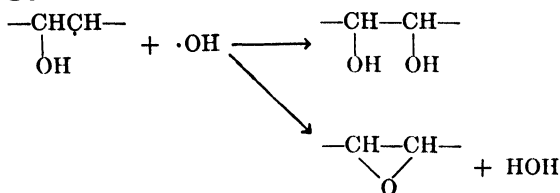
For the autoxidation at high temperatures which seems to involve more destruction of double bonds Waters (144) has proposed a different mechanism based on the observation that hydroperoxides decompose thermally yielding hydroxyl radicals. These radicals do not seem to be able to break the C—H bond but apparently add to the double bond:



This is then followed by short autoxidation chains giving two types of hydroperoxides which generate free hydroxyl radicals once more:

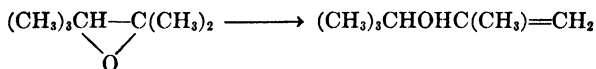


Other chain-breaking reactions would explain the occurrence of epoxides and α -glycols in autoxidation products:

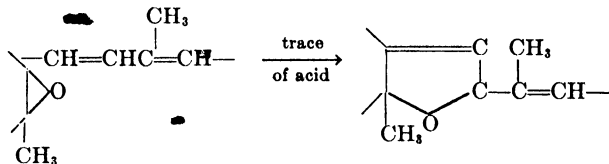


Further oxidation of these products could then cause chain fission at the site of the original double bond.

Recently Hickinbottom (64) has found that an epoxide readily rearranged to an unsaturated alcohol:



Furthermore, Karrer's work (88) on the polyene epoxides shows the great reactivity of certain epoxides:



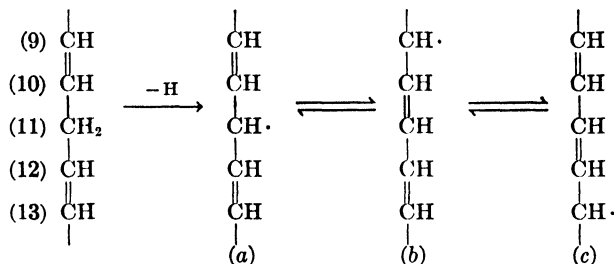
These illustrate some new possibilities for secondary rearrangements in the autoxidation products.

B. POLYETHENOID, METHYLENE-INTERRUPTED

Earlier work on the autoxidation of the unsaturated fatty acids containing methylene-interrupted double bonds will not be reviewed here. The recent discovery of the double-bond conjugation associated with the first step in the autoxidation of these compounds makes many of the analytical results (iodine number, refraction) difficult to interpret (48,49,52,137).

In 1943 Farmer and Sutton (46) observed that the autoxidation of unconjugated fish oil acids was accompanied by an increase in the absorption in the ultraviolet region at 230–240 mμ. The same observations had been recorded earlier, although the increased absorption had not been connected with the autoxidation (34). Farmer, Koch, and Sutton (42) then showed that in the earlier stages in the autoxidation of methyl linoleate and methyl docosahexaenoate a strong absorption band at 234 mμ appeared and increased parallel with the oxygen uptake and the peroxide formation in the earlier stages of the autoxidation. They explained these findings by assuming that the autoxidation takes place according to the free-radical mechanism proposed earlier by Farmer (42). The most reactive place would be the methylenic group between the double bonds at C-11 and C-14 in the case of linolenic acid. Abstraction of a hydro-

gen atom at one of these methylene groups would yield a free radical that would be stabilized through resonance with the three contributing structures *a*, *b*, and *c*:

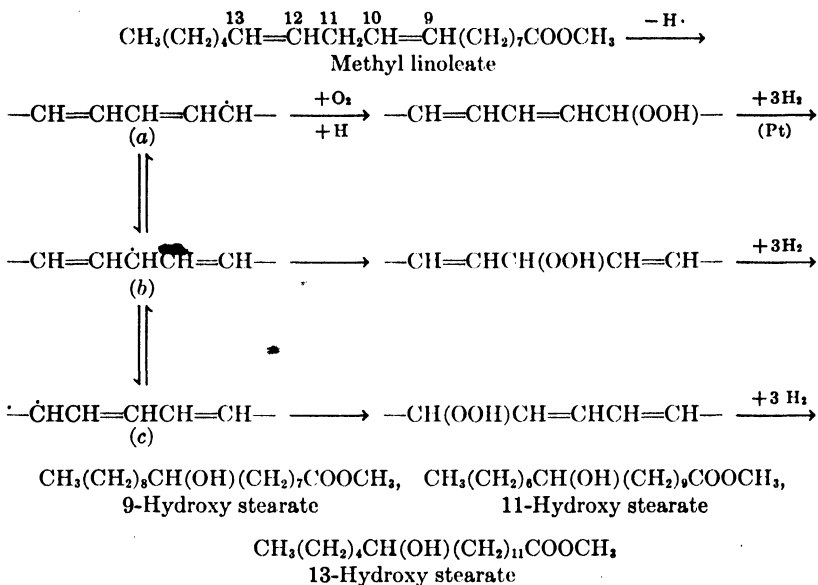


The radicals then react with oxygen in radical cycles as indicated earlier (page 431) yielding hydroperoxides.

The detachment of a hydrogen atom from the central methylene group of a 1,4-diolefin should be considerably easier than the removal of a hydrogen atom from an ordinary α -methylene group by virtue of the greater resonance energy of the former radical. Orr has calculated the resonance energies to be 30 and 19 kg.-cal. per gram mole, respectively. This appears to be the main reason for the higher rate of autoxidation of the 1,4-diolefins. The conjugation of the double bonds can only take place when the double bonds are separated by one methylene group. No conjugation is observed in the autoxidation of squalene or natural rubber, where the double bonds are separated by two carbon atoms.

In agreement with this, Bolland and Koch (21) found the same double-bond conjugation to occur in the autoxidation of ethyl linoleate at 45°C. They found a molecular absorption coefficient per absorbed oxygen at 231.5 μ of 22,700 in the beginning of the reaction when all oxygen was still present in peroxidic form. One mole of methane per mole peroxide was evolved in the active-hydrogen determination according to Bolland (16), taken by these authors as evidence that the peroxidic oxygen was entirely in the form of hydroperoxido groups. If hydrogen abstraction had occurred only at C-11 and if complete conjugation had taken place in every oxidized molecule, a molecular absorption per absorbed oxygen of about 30,000 would have been expected (89,106,141). They explained the low observed extinction by assuming an incomplete rearrangement of

the double bonds in the intermediate radicals (435) resulting in approximately 70% conjugation.

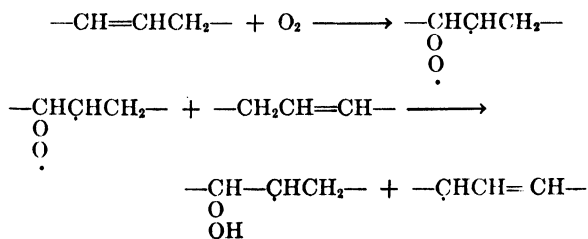


Independently Bergström (11,13) and Holman and others (79, 81-83) had made the same observations of spectral changes in connection with the autoxidation of linoleate. Bergström furthermore had hydrogenated the product obtained after autoxidation of methyl linoleate at 37°, and isolated and identified 9- and 13-hydroxy stearate by chromatographic separation on alumina. In order to facilitate the identification work Bergström *et al.* (14) synthesized the complete series of the seventeen isomeric monohydroxystearic acids. The stearates occurred in comparable amounts and constituted together the main reaction products of the hydrogenation. However, no 11-hydroxy isomer was found, although small amounts may have escaped detection, but there was always a certain amount of higher oxygenated compounds present. When samples of autoxidized linoleate with different oxygen content were hydrogenated there was always found about 0.2 mole of α -glycolic groups per mole oxygen absorbed in determinations with lead tetraacetate. Since all absorbed oxygen was present in peroxidic form, this could either indicate that a certain percentage of the primary hydroperoxides was rearranged

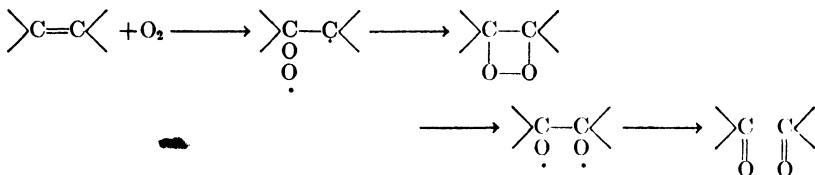
during the hydrogenation or that other reactions had already taken place in the early stages of the autoxidation, involving addition to the newly formed conjugated double bonds that would cause a decrease in the ultraviolet absorption. That a certain amount of secondary reactions does occur even in the earliest stages at various temperatures (40, 60, 80, and 100°C.) is shown by the work of Lundberg and Chipault (95). They found that a small fraction of the absorbed oxygen was not present as peroxide and this fraction increased with increasing temperature. They also found that secondary products absorbing at 277.5 mμ were formed in proportion to the oxygen uptake. Changes in the absorption upon the addition of alkali indicated that they were largely ketonic (81,82). See page 449.

Bolland (17) and Bolland and Gee (18,19) have made a careful kinetic and thermochemical analysis of the earlier stages of the autoxidation of ethyl linoleate under different conditions and all evidence was found compatible with the free-radical mechanism proposed by Farmer. Except in the very beginning of the autoxidation, the radical chains are initiated by thermal decomposition of the linoleate hydroperoxide. Kinetic evidence indicated the formation of one chain-starting radical from every two hydroperoxides decomposed. The oxygen uptake was found proportional to the peroxides present. Further evidence for the free-radical mechanism is that, as in other autoxidations, substances that are known to decompose and yield free radicals catalyze the reaction. Robertson and Waters (104) have suggested a mechanism by which the heavy-metal catalysts act, producing free radicals from the hydroperoxides.

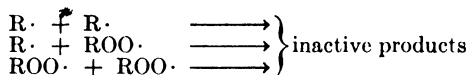
The mechanism by which the chains are started in the earliest stages might be somewhat different. It has been pointed out that more likely than a direct abstraction of a hydrogen atom at an α-methylenic group would be the direct reaction of oxygen with a double bond:



These two radicals would then start the radical chains discussed earlier, involving α -methylenic groups. However, the formation of a stable four-membered ring seems very doubtful, and it has been supposed that such a ring closure might lead to immediate rupture of the carbon chain (19,40,41):

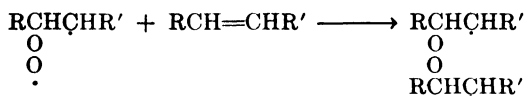


The most likely chain termination is the interaction of two radicals:

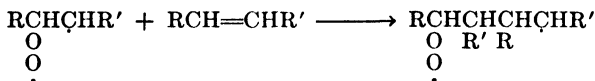


Since the average chain length in the autoxidation of linoleate at 45° was estimated at 100 it is clear that no conclusions can be drawn from the chemical evidence now at hand as to the chain-initiating or chain-breaking mechanisms.

The well-known tendency of concurrent polymerization in autoxidation of olefinic compounds is also readily explained by the free-radical mechanism. Any of the radicals might add to a double bond resulting in dimerization with an oxygen bridge:



The probability of this addition is greatest for conjugated systems, agreeing with the well-known polymerization tendency of these compounds. Furthermore a radical can add to a double bond before reacting with oxygen yielding hydrocarbon polymers:



For a summary of reactions of this type the reader is referred to the monograph of Price (100).

The reaction of the 1,4 diene system with *N*-bromosuccinimide and selenium dioxide has been investigated by Bergström (13a). When

bromosuccinimide reacts with methyl linoleate in carbon tetrachloride, a strong ultraviolet absorption appears at $232m\mu$, indicating a conjugation of the double bonds. On prolonged boiling the bromine is split off with the formation of a triene.

Since bromination with reagents of this type shows all the characteristics of a free radical reaction, these results lend further support to the autoxidation mechanism proposed by Farmer.

The discovery of the double-bond conjugation associated with the first steps of the autoxidation of linoleic and linolenic acids is of importance for understanding the reaction underlying the "boiling" or "blowing" and drying of linseed oil. When this oil, or the corresponding fatty acids, is heated in the absence of oxygen the double bonds are rapidly conjugated at 250°C ., but practically no conjugation occurs below 200° . This corresponds to the production of "stand" oil from raw linseed oil when heat conjugation followed by polymerization occurs. With strong alkalis the rearrangement occurs at somewhat lower temperatures (180 – 230°).

The "boiling" and "blowing" procedure is generally performed by blowing air through linseed oil at 120° with a small amount of added drier. Presumably no heat conjugation occurs at this temperature. However, the conjugation known to be a requirement for rapid and satisfactory drying is produced at this temperature by the double-bond rearrangement outlined above. What makes the "drying" of raw linseed oil so slow as compared with the drying of oils containing mainly fatty acids with conjugated double bonds (*i.e.*, tung oil, oiticica oil) is thus in part the time required for the formation of the primary peroxides with conjugated double bonds.

In the last few years our knowledge of the primary steps in the autoxidation of the unsaturated fatty acids has increased very much due largely to the work of Farmer, Gee, Koch, and associates in England. However, much less is known of the subsequent reactions when the peroxides decompose yielding the complicated mixture of more stable nonperoxidic compounds. Progress in this field has been hampered by the very difficult isolation problems involved. Infrared spectroscopy should prove of great value in this work.

IV. Action of Antioxidants and Their Biological Importance

The antioxidants of the phenol type (hydroquinone, pyrogallol, etc.) do not act by destroying the hydroperoxidic groups present (86),

but act through interfering with the radical chains in that they supply easily abstracted hydrogen atoms. The radicals then formed from the antioxidant are not reactive enough to carry on the chain by abstracting a hydrogen atom from an α -methylenic group. The semiquinones or aroxyl radicals formed are removed in pairs by disproportionation or recombination reactions. The autoxidation is thus kept under control by a chain-breaking mechanism as long as there are unchanged molecules of the antioxidant left (20,149-151). A similar mechanism involving the radicals is probably involved in the concomitant oxidation of a number of colored organic compounds. If compounds like polyenes, hemin, or dyes are present in autoxidizing unsaturated fats, these substances are rapidly decolorized. Haurowitz, Schwerin, and Yenson (60) have shown that hemin and hemoglobin, which are known to catalyze the autoxidation of unsaturated fatty acids, were rapidly oxidized to colorless products if added to linoleic or linolenic acid that was actively autoxidizing. Yenson (148) found the same for bilirubin, which also acted as an antioxidant.

Robertson *et al.* (105) found that thio ethers were oxidized under similar conditions, but in this case the peroxide itself could oxidize the thio ether to sulfoxide in the absence of oxygen. Similar reactions may be responsible for the rapid destruction of vitamin A, biotin, etc., when they are kept in an oil solution that autoxidizes. Holman (76a) found that the destruction of carotene in oxidizing fats occurs in the very beginning of the oxidation of the fat. Burr and Barnes (24) have reviewed the destruction of vitamins caused by the presence of unsaturated fats in the diet.

The destructions mentioned above seem to take place most readily when fatty acids of the 1,4-olefinic type (linoleic, linolenic, fish oil acids) autoxidize, whereas oleic acid or the unsaturated fatty acids with conjugated double bonds show a comparatively weak action. Except in the cases of the thio ethers, it is not the peroxides themselves that cause the oxidation, because no rapid decoloration takes place if the substance is dissolved in peroxide-containing oils kept under nitrogen. This indicates that it is a more reactive intermediate, probably a free radical, that causes the rapid decolorization—in principle, the same reaction as the destruction of the antioxidants discussed earlier. With the polyenes, however, the addition of the radicals, instead of hydrogen abstraction, might be the dominating reaction, causing the formation of cyclic and polymeric peroxides, chain breakage, etc.

Smith and Stotz (110) investigating the autoxidation of linoleic acid in aqueous suspension found it dependent on copper ions. The oxygen uptake could be inhibited by compounds forming complexes with copper ions. Hydroquinone also had inhibitive action in this system but showed evidence of differing in mechanism of action from the copper inhibitors. In this connection it might be pointed out that Bergström (9,10) has found the rapid autoxidation of cholesterol in aqueous colloidal solution to be catalyzed by copper ions and not by other heavy metals, indicating that copper ions have a specific action on autoxidations in aqueous suspension.

The antioxidants also seem to play a role in the living organism. Dam and Granados (31) found that in vitamin-E-deficient chicks and rats there was an increased peroxide content in the fat of several organs. It had earlier been shown by Burr and collaborators (8,26,56) that the fat from rats and hogs on a vitamin-E-deficient diet autoxidized more rapidly *in vitro* and did not show an induction period as did the fat from normal animals. The normal content of tocopherols in the fat depots of these animals seems to be derived entirely from the diet; the tocopherols are apparently the only antioxidants that are absorbed and deposited in this way.

V. Lipoxidase

A. HISTORICAL

Many of the early reports regarding the enzyme system now known as lipoxidase were made on what was erroneously considered to be a carotenoid oxidase. The bleaching of pigments by an enzyme present in legume seeds was first discovered by Haas and Bohn in 1927, and the use of soybean preparations to bleach the yellow pigments in wheat flour was patented in 1934 (57). This destruction of carotene by soybean preparations was later shown to be accompanied by the complete loss of its vitamin A activity by Frey *et al.* (50). Hauge and Aitkenhead (59) and Hauge (58) attributed the loss of vitamin A activity in dried alfalfa to an enzymic factor, later shown to be lipoxidase (97,98). Wilbur *et al.* (147) found that incorporation of soybeans in the diet of dairy cows led to the decrease in vitamin A activity of the butter produced.

In 1932 André and Hou (1) discovered a fat-oxidizing enzyme system in soybeans, to which the name lipoxidase was applied. A similar system in white beans was described by Craig (28) in 1936. In.

1939 Sumner and Dounce (122) studied the "carotene oxidase" system of soybeans and found that it led to the production of peroxides. It was shown by Sumner and Sumner (124), and later by Tauber (132), that carotene oxidase bleaches carotene only in the presence of oxidizing fat, and that the enzyme is really an unsaturated fat oxidase (126).

The subsequent work on lipoxidase will be discussed as it is related to the topics to be treated in this review. For detailed reviews of the early work on lipoxidase with impure preparations, the reader is referred to the papers of Süllmann (114) and Jezeski (87).

B. DISTRIBUTION IN NATURE

The occurrence of unsaturated fat oxidase is rather widespread in nature. The demonstration of these enzymes in soybeans, white beans, and alfalfa has already been mentioned. Strain (112) has detected lipoxidase in a variety of legume seeds, and Kirsanova (93) has found "carotene oxidase" in radish and potato juice. Sumner and Tressler (128) reported the activities of various soybean products, and Reiser and Fraps (101) listed the relative activities of numerous varieties of beans and peas. Süllman (113) was able to demonstrate lipoxidase in various parts of a variety of plants. Extracts of leaves and root nodules of several plants of the *Solanaceae* and *Labiatae* were found to be quite active.

Van Fleet (139,140) has studied the unsaturated fat oxidase systems in several plants using histochemical techniques. He reports that the oxidase activity is high at the seedling stage in plants grown on an alkaline medium, and that the oxidase is activated in regions of the plant that are neutral or alkaline, where water loss takes place, below wound surfaces, or where antioxidants are ineffective.

A study of the relationship between lipoxidase activity and fat composition in germinating soybeans has recently been made (77). The results indicate a decrease in lipoxidase activity after the second day from planting. Simultaneously, a preferential decrease was observed in the linoleic and linolenic acid contents of the fat. The decrease in enzyme after the initiation of the substrate oxidation may be caused by the inactivation of the enzyme as a consequence of its action, a well-known phenomenon *in vitro*. It may also be that the lipoxidase functions in initiating the autocatalytic oxidation of the linoleic and linolenic acids.

The presence of unsaturated fatty acid oxidases in animal tissues has been indicated in a few cases. Banks (5,6) demonstrated a heat-labile system in herring muscle which stimulated rancidity in the herring oil. Subsequent unpublished work by Banks (7) indicates that

this oxidase action involves hemin proteins and that their properties are quite unlike those of lipoxidase, having their optimum activity in the emulsified systems existing at low *pH* values. The system encountered in fish tissue has properties parallel to those of *in vitro* accelerated oxidation of linoleate in the presence of hemin (6). Lea (94) similarly demonstrated an oxidase system in pig muscle which accelerates rancidity in the fat and is most active at *pH* 4-5. Recent work by Watts and Peng (145a) indicates that lipoxidase activity of pig muscle extract is due to its hemoglobin or myoglobin content.*

Hove (84) found that rat liver and gastric mucosa contained an unsaturated fat oxidase, but intestine and muscle were inactive. Extract of gastric mucosa retained its activity at *pH* 3. Süllman (121) has demonstrated strong unsaturated-fat oxidase activity in striated muscle of the rabbit. Brocklesby and Rogers (22) reported that an extract of salmon liver destroyed 200 units of vitamin A in 45 minutes at room temperature, indicating the probable presence of an unsaturated-fat oxidase. None of these animal unsaturated fat oxidase systems has been well characterized as yet, nor have any purifications been reported. It is still not clear whether unsaturated fat oxidases play a significant role in animal metabolism.

Without doubt animal tissues contain catalysts for the peroxidation of linoleic acid, but no evidence to date indicates that the system involved is identical to soybean lipoxidase in its action. On the contrary, the meager evidence available points to the agency of some iron-containing system.

C. SUBSTRATE SPECIFICITY

It has been reported that lipoxidase can use as substrates those fatty acids or esters in which a double bond exists in the 9,10 position (111,112,125). Many of these and other tests (116,120) leading to conclusions regarding the substrate specificity were made on impure substrates, confusing the results obtained.

Using purified fatty acids and esters Holman and Burr (78) found that lipoxidase in crude extracts attacked linoleic acid, linolenic acid, their esters, and methyl arachidonate, and Holman and Elmer (80) showed that linoleate, linolenate, and arachidonate were oxidized at the same rate. From this work it is apparent that the posi-

* Reiser (100a) has prepared hemoglobin-free extracts of swine adipose tissue capable of decolorizing carotene in corn oil.

tion of the double bonds is not critical, because arachidonic acid is 8,11,14,17-eicosatetraenoic acid. However, they did show that only the *cis* isomers of linoleic and linolenic acids (the natural isomers) are attacked. The necessary substrate structure for attack by lipoxidase seems to be the methylene-interrupted, doubly unsaturated system, $-\text{CH}=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-$, with both double bonds *cis*. The possibility that conjugated unsaturated systems are attacked by the enzyme is doubtful (78,120).

D. STANDARDIZATION

A variety of methods of assay of lipoxidase involving different properties has been suggested. Sumner (127) developed a method for lipoxidase determination using linoleic acid suspension as substrate and the thiocyanate method for determination of peroxidic products. Reiser and Fraps (101) proposed a method based upon carotene destruction. Süllman (113), in his measurements on plant juices, used the uptake of oxygen as a measure of activity. Cosby and Sumner (27) used a lipoxidase assay in which the unit was the amount of enzyme which would cause the 50% destruction of carotene in 300 seconds under their prescribed conditions. More recently Sumner and Smith (123) used an assay based on the destruction of bixin, a carotenoid dicarboxylic acid. Balls, Axelrod, and Kies (3) also used carotene destruction as the basis of their assay.

All these methods of assay are subject to considerable error because the rate of oxidation is influenced by the degree of dispersion of the substrate. Those methods using carotenoid destruction are subject to the disadvantages that the rate of carotenoid destruction is proportional to enzyme concentration only over a narrow range, and that a side reaction, rather than the primary phenomenon, is being measured.

A method of assay developed by Theorell, Bergström, and Åkeson (134) overcomes these disadvantages. A homogeneous substrate solution is used, and the products of the reaction are measured either spectrophotometrically (the conjugated hydroperoxides absorbing strongly at 234 $m\mu$), or with the iron thiocyanate method. Under these conditions, peroxide production is proportional to time and to enzyme concentration over wide ranges. The details of the spectrophotometric methods are as follows:

One ml. of substrate containing 2 mg. linoleic acid in borate buffer at pH 9 is pipetted into the main compartment of a side-arm test tube. 0.2 ml. of enzyme

solution are pipetted into the side arm, the tube is filled with oxygen, and placed in a water bath at 20°C. The contents are mixed, and at the end of two minutes 2.0 ml. ethyl alcohol is added to stop the reaction. The mixture is diluted ten times with 60% alcohol, and the light absorption at 234 $m\mu$ is measured with a Beckman spectrophotometer. One unit of enzyme will develop a $\log I_0/I$ of 2.0 in one minute in the mixture (3.2 ml.) obtained after stopping the reaction with alcohol.

E. PURIFICATION

The first report of a purification of lipoxidase was made in 1943 by Balls, Axelrod, and Kies (3), who succeeded in purifying the enzyme 115 times over the water extract. Cosby and Sumner (27) later reported a sixtyfold purification of the enzyme. Süllman (119) was able to prepare stable acetone powders of the enzyme. Theorell, Bergström, and Åkeson (133) obtained an electrophoretically homogeneous preparation and these authors (134) later reported an improved method of preparation. In the same laboratory the pure enzyme was finally isolated and crystallized by Theorell, Holman, and Åkeson (135,136). The successful isolation of lipoxidase was carried out as follows:

15 kg. of defatted soya flour was suspended in 100 liters of 0.1 *M* acetate buffer at pH 4.5. The insoluble matter was removed in a basket centrifuge; the extract was adjusted to pH 6.7 with ammonia; and five volumes of 20% barium acetate, ten volumes of acetone, and two volumes of 20% basic lead acetate were added per 100 volumes of extract. The inactive precipitate was removed in a large separator. The inactive precipitate, formed upon the addition of 25 g. ammonium sulfate per 100 ml. extract, was allowed to settle, and the active supernatant fluid was decanted. Ammonium sulfate was added to bring the concentration to 40 g. per 100 ml., and the active precipitate was recovered. This was dissolved in a minimum of water and heated to 63°C. for five minutes to precipitate inactive protein. The supernatant fluid, containing 11.4 million units, was fractionated with ammonium sulfate, and the fraction between 35 and 50% saturation was kept. This was fractionated with alcohol in the cold in 0.02 *M* phosphate buffer at pH 5.5, and the fraction precipitating from 0 to 12% alcohol was taken. This was again fractionated with ammonium sulfate, and the fraction precipitating between 50 and 60% saturation yielded 1.8 million units having an activity of 358 units at 280 $m\mu$. This was subjected to electrophoresis in the large Tiselius apparatus, and 0.34 million unit of lipoxidase having 820 units per mg. was separated. This was concentrated and dialyzed against ammonium sulfate of slowly increasing concentration. The enzyme crystallized out in the form of colorless plates or sheaves. Washing away the small amount of amorphous material with less concentrated ammonium sulfate left crystals having an activity of 850 units per mg. This represents a purification of about seventy times that

of the buffer extract, or about 150 times that of a water extract of soy meal. It should be pointed out that the isolation is not strictly reproducible on different batches of beans, and that, therefore, exploratory experiments are necessary to determine the exact method of isolation.



Fig. 2. Crystalline soybean lipoxidase from ammonium sulfate ($\times 1200$).

F. PROPERTIES OF CRYSTALLINE LIPOXIDASE

The crystalline preparation of lipoxidase (135,136) has been shown to be homogeneous electrophoretically at pH 6.0 and 7.3, and it is also homogeneous according to sedimentation and diffusion patterns. The sedimentation constant and diffusion constant, determined by Dr. K. O. Pedersen of Uppsala, were 5.62×10^{-13} and 5.59×10^{-7} , respectively. The partial specific volume is 0.750. The molecular weight is thus 102,000. At pH 5.92 lipoxidase migrates anodically at a velocity of $\mu = 1.81 \times 10^{-5}$, and at pH 3.97 it migrates cathodically at a velocity $\mu = 4.63 \times 10^{-5}$. The isoelectric point is thus approximately at pH 5.4. These values agree very well with a mobility curve made with impure preparations.

The absorption spectrum of lipoxidase is that of a common protein with a moderately high absorption at 280 $m\mu$ ($\log I_0/I = 1.78$ for 1

mg./ml./cm.). No evidence of a prosthetic group is indicated by the absorption spectrum.

Iron determination has not been made on the pure enzyme, but the iron present in a 94% pure specimen would require a molecular weight of 370,000 for one atom iron per molecule. It is thus clear that iron could not be an active part of the molecule. This is in agreement with the inhibitor experiments.

Lipoxidase is soluble in dilute salts but insoluble in water. The break in its ammonium sulfate solubility curve comes at approximately 50% saturation. It is precipitated by about 2.3 *M* equimolar mono- and dibasic potassium phosphates, and fractionation of crude lipoxidase with this medium promises to be valuable in purification procedures.

Crystalline lipoxidase has a *pH* optimum (76) near *pH* 9, but the activity remains high up to *pH* 11.7. The temperature optimum lies near 30° (see Fig. 3) but activity decreases precipitously above that temperature due to a temperature-accentuated inactivation by contact with either substrate or reaction products. The *Q*₁₀ between 0° and 20° is about 1.6. Lipoxidase has a Michaelis constant for reaction with linoleic acid of 1.35×10^{-3} *M*, and for its reaction with oxygen 1.2×10^{-4} *M*. The enzyme is saturated with oxygen at 160 mm. O₂ pressure.

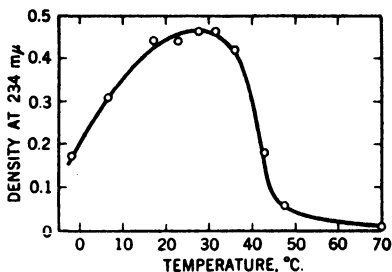


Fig. 3. Effect of temperature on lipoxidase activity. (Two minute assay at 20° C.)

G. INHIBITION

Several studies of the inhibition of lipoxidase (4,28,55,85,96,115, 118) have been made, but, perhaps because of the crude enzyme preparations and the various conditions used, there is little agreement in the reports. Polyphenols, however, are generally conceded to be inhibitors of the enzyme action. In studies of the action of the enzyme inhibitors on pure lipoxidase Holman (76) has shown that pyrophosphate, fluoride, cyanide, azide, mercury ions, *p*-chloromercuribenzoate, and diethyldithiocarbamic acid are ineffective as inhibitors even at high concentrations. Thus it is apparent that neither heavy metals nor sulfhydryl groups are the active centers in lipoxidase.

α -Naphthol and α -tocopherol have been found to be moderately inhibitory, but it is probable that this inhibition is due to phenolic

antioxidant activity rather than to true enzyme inhibition. Substrate competition is exhibited by elaidolinolenic acid, 10,12-linoleic acid, oleic acid, and octanoic acid, arranged in order of decreasing affinity for lipoxidase. See Figure 4 (76). As was expected, these

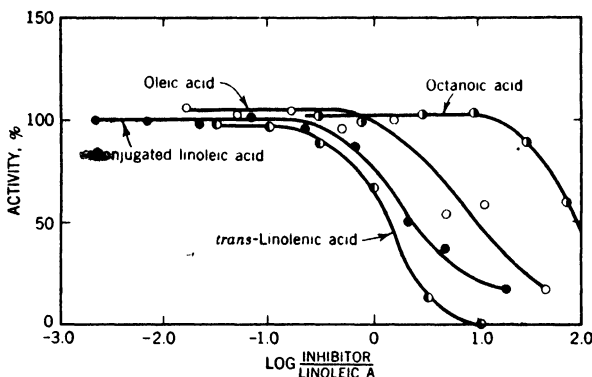


Fig. 4. Substrate competition for lipoxidase by fatty acids.

data indicate that lipoxidase is attracted by unsaturated centers in its substrates. They also indicate that there is a slight attraction for the saturated fatty acids. This phenomenon may have a function in decreasing the lipoxidase action in the plant when the substrate concentrations are low.

H. LIPOXIDASE ACTIVATOR

The work of Balls *et al.* (3) and Theorell *et al.* (133) on emulsified substrates shows that lipoxidase is activated by a polypeptide present in soybean and other sources (92). A polypeptide having the ability to increase lipoxidase action 300% under favorable conditions has been isolated and crystallized by Kies (91,92). However, in the single-phase sodium linoleate substrate, crystalline lipoxidase requires no activator. It is likely that the need for an activator demonstrated previously is a property of the emulsion systems used, and the activator probably has its action through some surface tension phenomenon. This is indicated by a variety of evidence.

In systems identical except for pH (and, consequently, the solubility of linoleic acid) the activity of crystalline lipoxidase approximately doubles when the pH is raised from 7 to 9. In unpublished

work Holman showed that organic solvents like ether, methyl acetate, and acetone can, in optimum concentrations, increase the uptake of oxygen 25 to 40% in emulsion systems. The maxima in the activity-concentration curves for ether and methyl acetate lie at concentrations of about 5% organic solvent, and the activity of the systems decreases precipitously when the water solubility of these substances is exceeded. Similarly, 0.2% sodium glycocholate increases lipoxidase activity 60%, and 0.4% sodium stearate can increase the activity 75%. The nonspecific surface tension effect shown in *in vitro* experiments may also operate *in vivo* where the substrate for lipoxidase may exist in insoluble form, but it does not operate in a single-phase system with pure enzyme.

I. REACTION MECHANISM

Bergström (12) and Holman (75) found evidence that the lipoxidase oxidation of linoleic acid follows the same course as autoxidation,

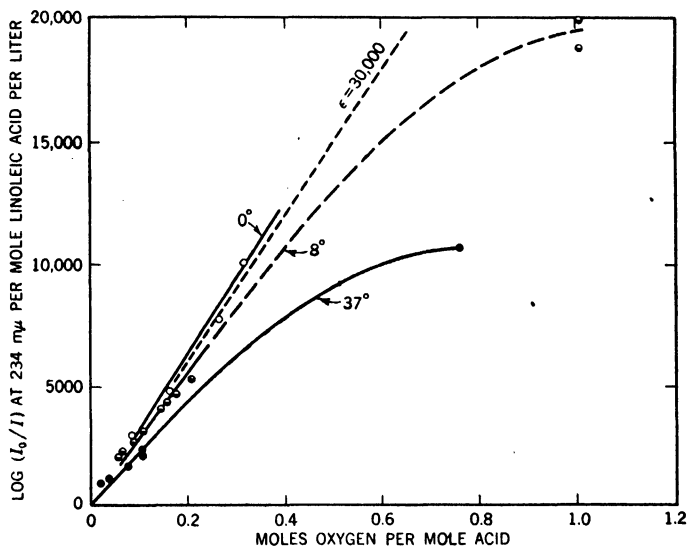
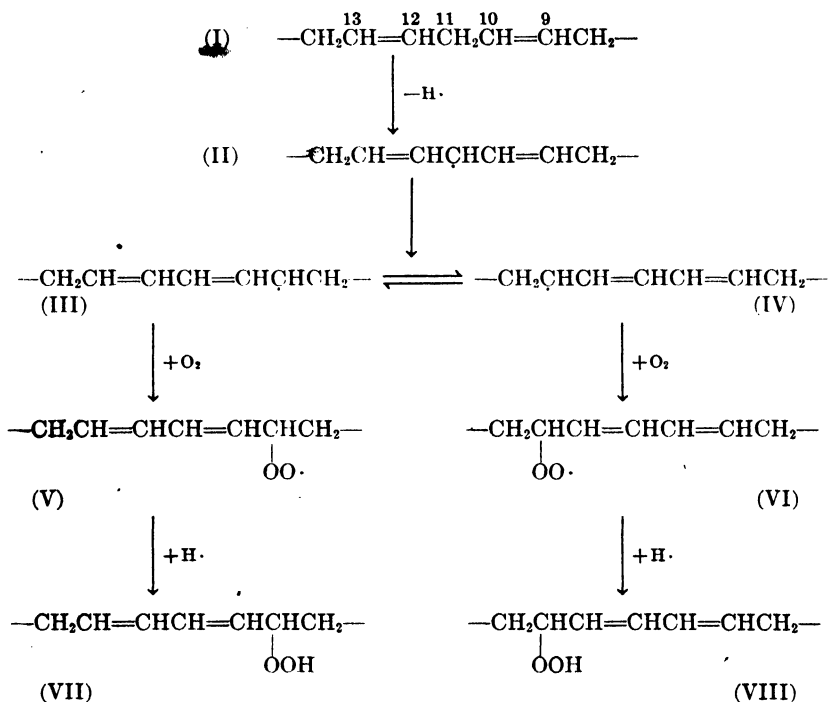


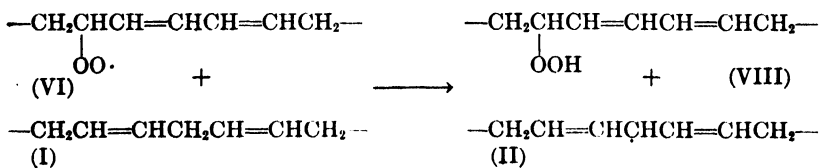
Fig. 5. Effect of temperature on degree of conjugation of linoleic acid oxidation with lipoxidase.

leading to hydroperoxides which are largely conjugated (pp. 435, 436). Recently it has been shown (15,76) that with homogeneous

substrate solution in low concentration, at 0°C., and with pure lipoxidase, linoleic acid is oxidized to yield totally conjugated peroxides. As the temperature is increased, the side reactions or subsequent oxidation or decomposition of the peroxides increase also, and the apparent degree of conjugation decreases from $\epsilon = 31,400$ at 0° to 23,000 at 37° (see Fig. 5)(76). In accordance with the evidence now at hand, under ideal conditions, the mechanism of reaction might be as follows:



The oxidation of linoleic acid, once started by the enzyme, could continue by means of a chain reaction. For example, the radicals V or VI could accept a hydrogen atom from linoleic acid:



This would give rise to another radical, II, which would go through the same cycle. Lipoxidase may thus have its function in initiating the chain reactions by removal of hydrogen from the linoleic acid methylene group. For a discussion of enzymic reactions as chain reactions see Waters (143,145).

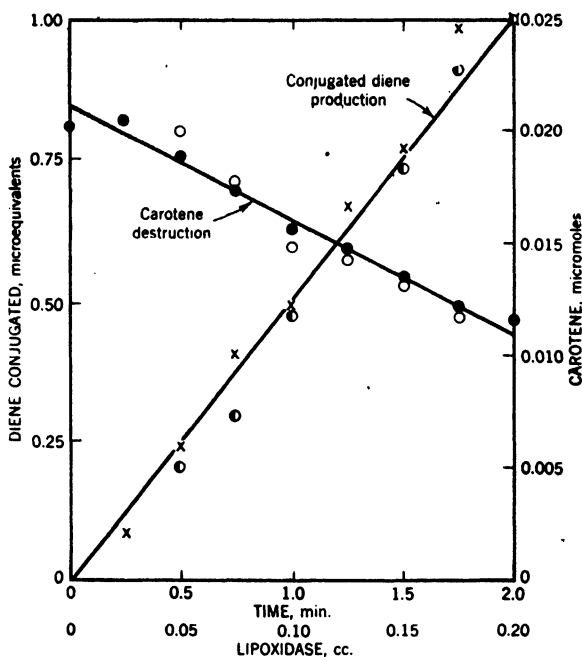


Fig. 6. Coupled oxidation of carotene and linoleic acid: (●, ×) enzyme concentration constant; (○, ◐) two-minute reaction time.

The carbonyl compounds detected among the oxidation products (75,117) produced under adverse conditions with crude enzyme are either decomposition products of the peroxides or products of side reactions induced by other enzymes present, for which Kies has found evidence (91).

J. COUPLED REACTIONS

The coupled oxidation of a variety of easily oxidized substances and linoleic acid under the action of lipoxidase has been studied by many

investigators. Among these substances are various carotenoids (111,112), chlorophyll (112), hemin (90), ascorbic acid (112), and dyes (112) (see page 441).

From a few data gathered on the coupled oxidation of carotene and linoleic acid using pure enzyme (77a), it has been found that the amount of carotene destroyed and the amount of diene conjugated

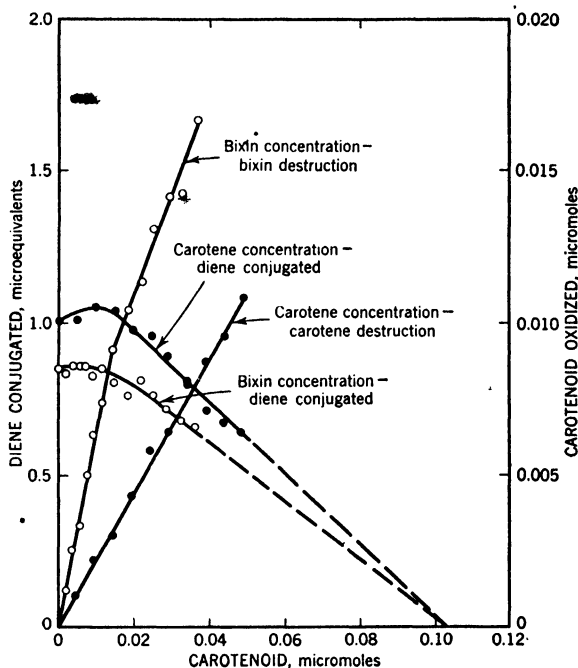


Fig. 7. Influence of carotenoid concentration on carotenoid destruction and diene conjugation.

are both proportional to the enzyme concentration and to time (see Fig. 6). Thus, in this system, carotene decomposition is a good measure of the degree of oxidation, and can be used as a measure of the enzyme activity. In studies with both carotene and bixin (Fig. 7) it has been demonstrated that carotenoid destruction during the reaction is proportional to the carotenoid concentration. It will be seen from curves 1 and 3 in Figure 7 that, as the carotenoid concentration is increased, the degree of production of conjugated diene is reduced, and that the oxidized carotenoid is also increased. From the slopes

of these two sets of curves it can be calculated that the oxidation of one mole of carotene prevents the formation of 43 moles of conjugated diene, and that one mole of bixin prevents the formation of 26 moles of diene.

It appears that the coupled oxidation of carotenoids, and perhaps other substances, occurs as a consequence of their interruption of the chain oxidation of linoleic acid. The radicals V or VI may accept hydrogen atoms from carotene, leading to its destructive oxidation and the termination of the chain reaction. The efficiency with which the carotenoids prevent the formation of conjugated diene varies, and it is possible that antioxidants (substances which can break chain reactions) vary in activity according to the affinity they have for the free radicals or the ease with which hydrogen is abstracted from them.

Further investigation will be required before the mechanism of this enzymic oxidation of linoleic acid and its coupled oxidations in aqueous solutions can leave the realm of speculation.

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ENZYMES OF SNAKE VENOMS AND THEIR BIOLOGICAL SIGNIFICANCE

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I. Introduction

Snake venom is a mixture varying in composition from species to species. Furthermore the poisonous material from a single species, collected from different geographical areas or taken from animals under different physiological conditions, can show considerable variation.

Several enzymes have been found in snake venoms, one of the most

potent sources of these biological catalysts. The question arises whether these enzymes play a role of any importance in the syndrome produced by the poisons in the bitten animal. There is no doubt that this can be answered in the positive. But our knowledge is still very limited. Far more quantitative data than are available today on the enzyme activity of various snake venoms must be accumulated to add to the progress in this field. The results obtained should be compared with the corresponding biological activities of the venoms. The results of the intensified work on the pharmacological behavior of isolated and purified enzymes will also help (40). But it must be borne in mind that some components of the venoms profoundly influence the reaction mechanism of the others.

In the first sections of this review, only those enzymes are fully described which have been investigated since the article of Kellaway appeared in 1939 (68). In discussing the biological significance of the enzymes of snake venoms, certain other enzymes are considered.

In 1881, de Lacerda described the proteolytic activity of the venom of a *Bothrops* species (72). But even before this time the opinion was expressed that agents similar to the enzymes of the digestive juice may occur in the poisons (104). The results of de Lacerda were confirmed and extended by numerous workers. The investigations of Lamb (73), Martin (85), and Calmette (101) on the coagulation of blood contributed a great deal to the knowledge of the snake venom proteases.

Another trend of nineteenth century origin led to the discovery of a group of enzymes responsible for hemolysis and similar phenomena. Delezenne showed that an enzyme of venoms, now called lecithinase A, converts lecithin to lysolecithin, the agent responsible for hemolysis in bitten animals (29). The same author has shown the existence of another group of enzymes. He observed the disintegration of nucleic acids and the production of nucleosides in the presence of snake poisons (30). However, this and other phosphatases of the venoms were not fully investigated until almost twenty years later.

These results and many others led step by step to the conclusion that enzymes are more than accidental components of snake venoms. A comprehensive article by Houssay in 1930 (61) reviewed the experimental work on the rich enzyme pattern of the venoms, to which he himself had contributed greatly. But even then enzymes were not considered the real toxic principles of the venoms. During the fol-

lowing years much experimental work was accomplished to elucidate the role of some enzymes in the production of shock, hemorrhage, hemolysis, and blood clotting. Kellaway, in his summary of the results (68), mentions several enzymes at length as toxic principles.

II. Cholinesterase

In 1926 Abderhalden described the hydrolysis of acetylcholine in the intestine of horse and pig (1), a reaction foreseen by Dale (27). In the same year, in his studies on the transmission of nerve impulses by chemical substances, Loewi found that an agent present in heart extracts inactivated acetylcholine (77). Some years later he established that the enzyme-like nature of the heart extract component was responsible for the disappearance of acetylcholine (39). Stedman, working on horse serum and pig liver esterases, confirmed the high acetylcholine-destroying activity of serum (120) found by Plattner and co-workers some years before (106,107). Inhibition experiments led them to assume that the enzyme involved was an esterase; accordingly they called it cholinesterase (ChE).

A. TYPES OF CHOLINESTERASE

Since horse serum hydrolyzes not only acetylcholine but also ethyl acetate and tributyrin, it could not be decided "whether one enzyme is responsible for the hydrolysis of all three types of substrate, or whether different enzymes are involved" (120). In the course of their studies, Stedman and his co-workers compared the acetylcholine-splitting capacity of several sera and performed experiments on competitive inhibition with acetylcholine, tributyrin, and ethyl butyrate. Although these workers reached the conclusion that ChE has a specific action on choline esters they admitted the probability of the hydrolysis of other esters, for instance, tributyrin, by the same agent. Contrary to the results obtained with human serum they found a second esterase in horse and guinea pig sera, distinguished from human esterase by its insensitivity to eserine (35). These authors even mentioned the possibility of hydrolysis of choline esters by the "second esterase." Thus the question arose whether more than one enzyme is able to inactivate acetylcholine.

These results indicated a relationship between the enzymic hydrolysis of acetylcholine and esterase action on substances like methyl butyrate, ethyl acetate, tributyrin, etc. These enzymes have been

known since the beginning of this century and were thoroughly investigated in 1920-1932 by Rona, Willstätter, and their co-workers and pupils (5). Owing to the inadequate concept of enzyme specificity in the thirties, these relationships sank into almost complete oblivion. Only Vahlquist published results to show that ChE was not specific for choline esters (126). In the meantime, an enormous number of facts on ChE had accumulated (6,130). Special attention was given to various choline esters in their behavior toward this enzyme, and to the many dozens of substances inhibiting it (7).

The problem of ChE specificity was then discussed by Alles and Hawes (2,3). These authors noticed great differences between the cholinesterases of human serum and erythrocytes. Some of their results are summarized in Table I. They did not, however, continue their research along this line, and the question whether one or two enzymes are involved was left unsettled. Whether similar differences could be found in other tissues and species was also left unanswered. However, a firm basis for further development was established and two methods were introduced which were afterward extensively used by other workers, namely, inhibition by high substrate concentrations, on the one hand, and degradation of acetyl- β -methylcholine on the other. Shortly afterward, in 1942-1943, results from English (108), Canadian (89), and Swiss (134,147) laboratories led to the final conclusion that at least two different enzymes exist which are capable of hydrolyzing acetylcholine.

TABLE I
SOME PROPERTIES OF THE CHOLINESTERASES OF HUMAN SERUM
AND ERYTHROCYTES

Property	Serum ChE	Erythrocyte ChE
Optimal pH	8.5	7.5 to 8.0
Activation by NaCl	0	+
Inhibition by high substrate concentrations	0	+
	(optimum $>0.25 \times 10^{-4}$)	(optimum 0.25×10^{-6})
Hydrolysis of acetyl- β -methylcholine	0	+
Hydrolysis of tributyrin	+	0

Richter and Croft (108) investigated the blood ChE of several species and confirmed and extended the results of Stedman and other workers. In the majority of the sera studied an enzyme was found which was capable of splitting esters like methyl butyrate and tri-

butyryl, but not acetylcholine. This enzyme was named *ali*-esterase (for the aliphatic esters used as substrates). On the other hand, the acetylcholine-splitting enzyme of human serum is not specific for choline esters alone, since it attacks the substrates mentioned above as well. The ChE of human red cells appeared to be far more specific, failing to act on noncholine esters. From these and other results the authors concluded that ChE is not to be regarded a single entity.

Experiments undertaken with other than human blood were less conclusive, since red cells split not only the acetylcholine, but also noncholine esters. This point was then cleared up by Mendel and co-workers (89). These authors used purified preparations of erythrocyte ChE which, regardless of the origin of the cells, were unable to split methyl butyrate or tributyrin. The results obtained with highly purified serum ChE confirmed those of Stedman, Vahlquist, and Richter.

In addition to the substrates hitherto known, benzoylcholine was found to be hydrolyzed by serum ChE, but not by erythrocyte ChE (87); the reverse was true for acetyl- β -methylcholine (see Table I). It should be borne in mind, when benzoylcholine is used for diagnostic purposes, that enzymes which fail to attack acetylcholine may be capable of hydrolyzing the benzoyl ester (15,51,114).

TABLE II
INHIBITION EXPERIMENTS WITH CHOLINESTERASE OF HUMAN SERUM,
ERYTHROCYTES, AND BRAIN

Inhibitors		Per cent inhibition of ChE of		
Compound	Conc., <i>M</i>	Serum	Erythrocytes	Brain
Percaine ^a	0.006	94	25	12
Irgamide ^b	0.006	46	4	3
4-Isopropylantipyrine	0.002	65	9	18
Morphine	Saturated	66	76	66
Caffeine	0.006	4	42	40

^a Nupercaine, 2-butoxy-*N*(2-diethylaminoethyl)-cinchoninamide hydrochloride.

^b *N*¹-seneciocylsulfanilamide.

ChE of dog pancreas (86) behaved very much like that of serum; brain ChE resembled the red cell enzyme in its action. The ChE from serum and dog pancreas therefore seemed to be less specific

and was named "pseudo" ChE, while the enzyme of erythrocytes and brain received the name "true" ChE (89).

Many experiments were undertaken by Zeller (131,132,134) to elucidate the mechanism of ChE inhibition. In many cases the degree of inhibition varied when the (unpurified) enzymes were taken from different tissues (131,132). Thus, human serum ChE was much more sensitive to 4-isopropylantipyrine than the enzyme of human central nervous system (134). Working with Bissegger (147), we tried to throw some light on these phenomena. Some of the results of these investigations are recorded in Table II.

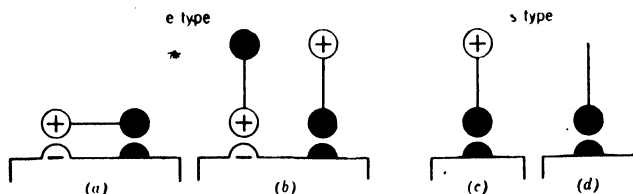


Fig. 1. Models of e- and s-ChE (147). Black circles: ester group; black half-circles: enzyme linked to ester group; white circles: positively charged group of choline esters; white half-circles: negatively charged group of ChE. (a) normal linkage of e type. (b) form of linkage produced by excessive substrate concentration. (c) linkage of acetylcholine with s type. (d) linkage of aliphatic esters (*e.g.*, methyl butyrate) with s type.

There is no doubt that the cholinesterases of human brain and erythrocytes are always inhibited to the same degree, while serum ChE usually shows very different behavior. Further analysis of the properties of these enzyme reactions led to the assumption that two enzymes are involved. Tentative models of the catalysts are sketched in Figure 1 (147).

This method of determining the type of acetylcholine-splitting enzyme by the use of inhibitors was afterward employed in many laboratories. Some of the procedures now available for the differentiation of cholinesterases are summarized in Table III.

The two enzymes were named s- and e-ChE (147). The designations serum- and erythrocyte-ChE were deliberately avoided, since we knew from the beginning that the two types were present in other tissues as well. The letters e and s are reminders of the first source

(human blood) of the respective enzymes. This notation enables the use of similar names if new types are found.

There is no doubt that many of the differences among the cholinesterases of various tissues cannot be accounted for by the fact that two types exist. Some of the variations may be due to the protein moiety of the enzyme involved. It is often noticed that pure enzymes behave differently in several respects when isolated from different sources. ChE proteins may well be specific not only with regard to species, but also with regard to organs.

TABLE III
METHODS OF DETERMINATION OF CHOLINESTERASE TYPES

Reaction	s type	e type	Ref. Nos.
Hydrolysis of benzoylcholine	+	0	87
of acetyl- β -methylcholine	0	+	2,87
Inhibition by high substrate concentrations	0	+	2,86,147
by pyrazolones	+	0 ^a	134,147
by local anesthetics (percaine)	+	0 ^a	98,147
by sulfonamides	+	0 ^a	131,132,147
by methylhydroxy purines	0	+	98,147
by triorthocresyl phosphate	+	0 ^a	90
by diisopropyl fluorophosphate (DFP)	+	0 ^a	59
by dimethyl carbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide	+	0 ^a	57,58

^a This represents a sensitivity markedly inferior to the inhibition type as compared with the other type.

Studies on human, horse, and guinea pig serum ChE revealed (135) various degrees of inhibition, due to corresponding affinities of the ChE to inhibitors (sulfonamides, percaine, and papaverine, a substance used for the first time in similar investigations by Langemann, 74) and to substrates. These studies were performed with the s enzyme only.

Hawkins and Mendel (58) found less inhibition of *Planaria* ChE with eserine and other amines than of human red cell ChE. Yet in other respects the two enzymes were more alike, so that *Planaria* ChE was regarded a "true" ChE. Even greater difficulties of classification were encountered when the properties of snail ChE were investigated by Augustinsson (8). In view of these complications this author concluded that "the problem of the specificity of cholinesterase is not solved with the hypothesis of two types of cholinesterases."

T A B L E I V
SOME PROPERTIES OF CHOLINESTERASE FROM DIFFERENT SOURCES^a

Source of ChE	Designation	Hydrolysis of				ChE inhibition by		
		Acetyl- β -methyl-choline	Benzoyl-choline	Methyl-butyrate or ethyl acetate	Tributyl ^b in	Excessive substrate concentrations	Eserine	Nu-489 ^s
<i>Rana pipiens</i> , brain <i>Planaria dorotocephala</i> <i>Helix pomatia</i> , dart sac blood	s type	0	+	+	+	0	+	+
	e type	+	0	0	0	+	+	\pm ^c
	"True" ChE	+				+	\pm ^d	
	"True" ChE	+	0	0	+ ^e	0	\pm ^d	\pm ^f
Snake venoms			\pm	0	+	0	+	
	c type	+	0	+	+	+	+	+

^a Dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium-bromide (Hoffmann-LaRoche Nu-489).

^b Diisopropyl fluorophosphate.

^c e type is less sensitive to DFP than s type.

^d These ChE preparations are less sensitive to eserine than e-ChE.

^e This effect of *Planaria* extract on tributyrin is not due to ChE.

^f *Planaria* extract is less sensitive to Nu-489 than e-ChE.

As will be seen later on, investigations on ChE of snake venoms lead to similar conclusions.

In Table IV are summarized some properties of the cholinesterases investigated so far. This record shows clearly that few properties are consistent. Even inhibition by excessive substrate concentrations is not always connected with the e type; but the s type is never associated with this kind of reaction. Possibly the substrate concentration in the e type reaction was not always high enough to display a velocity decrease.

Some of the principles of nomenclature hitherto used do not appear adequate to classify the increasing amount of data collected. The effect of "specific" ChE is not confined to choline esters only. The "unspecific" ChE, or "unspecified," esterase (97) probably is as specific as the other type, unless an enzyme which combines with more than one group of substrates is considered more specific than another which needs only one group. Objections were raised from the beginning (4,76) against the terms "true" and "pseudo" ChE. Indeed until more is known about the action of the enzymes and of their *in vivo* substrates, a less specific nomenclature is to be preferred.

We may conclude that more than two types of ChE exist, each represented by several related enzymes.

B. MEASUREMENT, OCCURRENCE, AND BIOLOGICAL SIGNIFICANCE OF CHOLINESTERASE

Estimations of ChE activity by biological assay of residual acetylcholine have generally been abandoned (for literature, see Alles and Hawes, 2). The same may be said of the continuous-titration methods based on acetic acid liberation, unless a glass-electrode potentiometer is used (2,48,112). The manometric methods depend on carbon dioxide liberation from bicarbonate buffer solutions by acetic acid. This procedure, although less precise than the potentiometric method, is well suited for serial determinations.

In this review only few data can be presented regarding the distribution of the different cholinesterases in the animal organism. An exception is made in the case of salivary glands and the nervous system, since the ChE of snake venom may interfere with the acetylcholine-splitting enzyme of brain and nerves.

The different parts of brain show very characteristic ChE values,

as has been shown by Nachmansohn (94), Pighini (105), Birkhäuser (10), and Zeller (143) in human, elephant, ox, dog, and rabbit brain. The ratio of the activities displayed by the different parts of the brain is higher than 20. The highest values are encountered in the central grey matter (*e. g.*, putamen), the lowest in the cortex and white matter. The differences are of the same order when the ratio of ChE and monoamine oxidase is used for comparison (10,75). (The latter enzyme inactivates adrenaline and other monoamines.) The *e* type is present in the brains of all species hitherto investigated (58,88,89,97, 143,147). On the other hand, the *e* and *s* types have been encountered in peripheral nervous tissue (116). The *e* type appears to be predominant in muscle (74).

Nachmansohn's work on the distribution of ChE in nerves indicates that the enzyme is located at the neuron surface (95). A very high concentration of ChE is present in the motor end plates of the neuromuscular junctions and in the synapses. But "there is only a quantitative difference between axon and synapse, the enzyme concentration being higher at the synapse where the neuronal surface increases due to the extensive end-arborization" (95).

It is assumed in this review that the classical theory on the role of ChE in the transmission of nerve impulses in synapses and motor end plates is generally known. Nachmansohn proposed a new concept. According to this author acetylcholine is released not only at the nerve endings, but everywhere on the neuron surface. This is related to the electrical changes during activity and consequently to the propagation of the impulse along the nerve fiber (95).

Naturally, the *e* type in the red cells can be only indirectly related to the mechanism of nerve action. This and other facts are consistent with the assumption of a metabolic function of *e*-ChE. Because of the complete lack of evidence regarding the true substrates of the *s* type, our knowledge of biological role of this enzyme, which is present in serum, dog pancreas, human ovary (74), etc., is rather limited. The enzyme could not be detected in the serum and tissues of ruminants (51). A group of Swiss workers found that the activity of the *s* type in human and guinea pig sera and in rat and mouse livers is influenced by certain sex hormones (11,128,144-146). Their results have been confirmed and extended by other authors (41,115).

Salivary glands contain different types of ChE. In the parotid glands of pig and guinea pig, *s*-ChE was present; in the parotids of

rabbit and cow, e-ChE was found, but a mixture of both types was detected in dog and cat parotids (51,88,114).

C. OCCURRENCE OF SNAKE VENOM CHOLINESTERASE

In 1938 Jynegar, Sehra, Mukerji, and Chopra (65) found that cobra venom contains an acetylcholine-inactivating enzyme. Most of the earlier work on the poison ChE was accomplished by Indian authors who had large amounts of cobra venom at their disposal.* The presence of ChE in snake venom awakened great interest because of the possible relationship to the neurotoxic principle of cobra poison. This venom is a hundred times more active than the enzyme of the electric organs of certain fish, hitherto considered the most active source of the enzyme (84,96).

ChE is found not only in cobra venom but also in the toxic secretions of *Bungarus fasciatus* (25,47). However, the enzyme was not detected in the venom of *Crotalus terrificus* (47), *Echis carinatus* (47), *Vipera russelli* (47,65), and *Vipera aspis* (149). From these results we concluded that the presence of ChE may be a characteristic difference between the venoms of *Colubridae* and *Viperidae*. With this in mind, a large series of venoms was investigated. Although none of the viperids contained ChE, it was found in all colubrids with one exception: a sample of *Enhydrina schistosa* venom, which was not very pure and more than fifty years old, did not hydrolyze acetylcholine. (The group of sea snakes to which *Enhydrina schistosa* belongs differs in many respects from the rest of the colubrids.) The viperid species examined, which proved to be negative are: *Agkistrodon mokasen*, *A. piscivorus*, *Bitis arietans*, *B. gabonica*, *Bothrops alternatus*, *B. atrox*, *B. cotiara*, *B. jararaca*, *B. jararacussu*, *B. itapetiningae*, *B. neuwiedii*, *B. nummifera*, *Crotalus adamanteus*, *C. cinereus*, *C. horridus*, *C. lucasensis*, *C. ruber*, *C. terrificus*, *C. terrificus basilicus*, *C. viridis*, *C. viridis oreganus*, *Echis carinatus*, *Sistrurus catenatus*, *Trimeresurus gramineus*, *Vipera ammodytes*, *V. aspis*, *V. aspis* from the Département du Gers (France), *V. aspis hugyi*, *V. lebetina*, and *V. russelli* (141).

The ChE of cobra and *Bungarus fasciatus* venom was purified considerably by fractional precipitation with sodium and ammonium sulfate. The increase in activity, determined by the ratio of ChE to

* Because it was not possible to secure a complete list of the original papers, part of this section has been reviewed from abstracts.

nitrogen content of the original and of the purified material, was more than twentyfold in *Naia naia* and more than elevenfold in *Bungarus fasciatus* (24,25). Considering the high ChE activity of many colubrid venoms (Table V), it is not surprising that these preparations are among the most active cholinesterases hitherto known.

D. SOME CHARACTERISTICS OF VENOM CHOLINESTERASE

The ChE of cobra and banded krait (*Bungarus fasciatus*) is heat labile, since crude and purified preparations lose their activity after being heated to 60–70° (47). The enzyme is capable of hydrolyzing not only acetylcholine, but also acetyl- β -methylcholine, the ratio of the reaction velocities of the two substrates varying between narrow limits (Table V). Unlike acetyl- β -methylcholine, benzoylcholine was not attacked. Cobra venom, on the other hand, hydrolyzed several noncholine esters (18). The reaction velocity is inhibited by high

TABLE V
CHOLINESTERASE IN THE VENOM OF *Colubridae*

Species	Q _{ChE} ^a	Q _{MCh} ^b	$\frac{Q_{ChE}}{Q_{MCh}}$	Ref. No
<i>Acanthophis antarcticus</i>	9240	7080	1.3	141
<i>Bungarus coeruleus</i>	24900	15300	1.6	141
<i>Bungarus fasciatus</i>	18700	22000	0.9	25,47,141
<i>Demansia textilis</i>	140			141
<i>Dendraspis angusticeps</i>	250			143
<i>Denisonia superba</i>	11000	9000	1.2	141
<i>Denisonia superba</i> var. from higher altitudes	3300			141
<i>Elaps corallinus</i>	680			141
<i>Naia bungarus</i>	4800	4740	1.0	141
<i>Naia flava</i>	7200			141
<i>Naia haie</i>	> 1020			141
<i>Naia melanoleuca</i>	27900	20000	1.4	141
<i>Naia naia</i>	> 13000			18,19,24,25, 47,65,113, 141,143,153
<i>Naia nigricollis</i>	40			141
<i>Notechis scutatus</i>	3300	2900	1.1	141
<i>Notechis scutatus</i> var. <i>niger</i>	4260			141
<i>Notechis scutatus</i> , white venom	3180			141
<i>Pseudechis australis</i>	90			141
<i>Pseudechis porphyriacus</i>	140			141
<i>Sepedon haemachates</i>	6750	4360	1.5	141

^a Q_{ChE} = microliter carbon dioxide per milligram dried venom with acetylcholine.

^b Q_{MCh} = microliter carbon dioxide per milligram dried venom with acetyl- β -methylcholine.

substrate concentrations (153), eserine, diisopropyl fluorophosphate (18), caffeine, and morphine (143,153). Some of these results are summed up in Table IV (page 466).

From these results it can be concluded that the characteristics of the e type are predominant. But it was already noticed some time ago that cobra ChE had certain peculiarities setting it apart from the e type of erythrocytes and brain (153). As mentioned previously, the salivary glands of mammals contain either e- or s-ChE, or a mixture of both. Therefore it cannot be ascertained whether the venom glands, which are homologous with salivary glands, produce the e or s type.

Since cobra venom hydrolyzes several noncholine esters, Bovet-Nitti suggested the name "acetylase" for the enzyme. But the experimental results so far reported did not make clear whether choline and noncholine esters were attacked by the same venom enzyme. Recently, we have been able to show beyond doubt that this question can be answered in the positive. The "*ali*-esterase" displays the same distribution among snake venoms as the ChE, and the ratio of velocities of the two substrate groups varies but slightly. The degradation of noncholine esters is inhibited by eserine in the same degree as acetylcholine. Simultaneous addition of both substrates leads to a competitive inhibition. For these experiments we used α -halogen acetic acid esters, which performed suitably in our enzymological analysis (143).

Contrary to the results mentioned above these latter results point clearly to the s type. Therefore it seems to be impossible to decide whether the venom ChE belongs to the s or e type. We must regard this enzyme as a new type of ChE. It may be well to use the name ophiocholinesterase, or, since this ChE is present only in colubrid venoms, to call it coluberecholinesterase (c type) (*coluber* = snake, Latin).

III. Hyaluronidase

Duran-Reynals, in 1936, found an agent in snake venoms which enhances the spreading of dyestuffs and infectious agents in the skin of animals (31,33). This author described the "spreading factor" for the first time in 1928. It is present in extracts of mammalian testes and leech heads, many invasive bacteria, venom of bees, scorpions, and poisonous fish, and in *Ankylostoma duodenalis* (32).

Chain and Duthie showed that the spreading factor of two snake venoms and of many other sources is identical with the enzyme which depolymerizes hyaluronic acid (23); this has been confirmed by numerous workers (32,91). In 1937 this hyaluronidase was detected in autolyzates of pneumococci by Meyer, Dubos, and Smyth (92). Meyer and Palmer three years earlier (93) had described hyaluronic acid as a new type of mucopolysaccharide which appears to bind water in the interstitial spaces and to hold cells together in a gel. "Hyaluronic acid, while allowing metabolites to pass through it, has the important function of offering resistance to penetration by foreign matter including agents of infectious diseases" (119). This inhibition breaks down completely under the action of hyaluronidase.

A. CHEMISTRY AND OCCURRENCE OF HYALURONIC ACID

Hyaluronic acid consists of equivalent amounts of *N*-acetyl-D-glucosamine and D-glucuronic acid. The basic unit seems to be an aldobionic acid with a free aldehyde group in the *N*-acetyl-D-glucosamine part (91). The molecular weight is at least 200,000 to 500,000 (16,17). Unlike the related chondroitinsulfuric acid, the polysaccharide does not seem to have a branched chain. Hyaluronic acid occurs either free or in the form of a salt-like compound with protein, from which it is often difficult to separate (91).

Hyaluronic acid has been found in the vitreous and aqueous humors, in the umbilical cord, nucleus pulposus, synovial fluids, and in the skin (91).

B. HYALURONIDASE, A MIXTURE OF SEVERAL ENZYMES

The *in vitro* depolymerization of the substrate by the enzyme occurs in a sequence of several phases. In the first phase we find a disappearance of clotting with acetic acid and proteins (79); in the second there is a sharp drop in viscosity, and in the last phase reducing substances and *N*-acetyl-L-glucosamine are liberated. If hyaluronic acid is bound to proteins, the linkages with the proteins have to be split in the course of this sequence of reactions. It seems unlikely that all steps of depolymerization are accomplished by the same enzyme. It has been claimed that two enzymes have been obtained by the fractionation of hyaluronidase (55). The first, called mucopolysaccharase, depolymerizes the substrate to the stage of aldobionic acid; the second, mucooligosaccharase, is said to split the aldobionic

acid unit. On the other hand, β -D-glucosaminase is found in several sources of spreading factors. This enzyme has been separated from the viscosity-lowering principles.

In summary, the complex of hyaluronidase of different origins undoubtedly contains different enzymes, but their number and mode of action are not fully known (91). The term hyaluronidase therefore does not designate a single entity, but a whole group of enzymes. The composition of this mixture varies with different organisms. For this reason it is impossible that all spreading factors and hyaluronidases are completely identical.

C. SOME PROPERTIES AND THE MEASUREMENT OF HYALURONIDASE

Hyaluronidase acts on hyaluronic acid, on the sulfuric acid ester of hyaluronic acid of the cornea, and on the chondroitinsulfuric acid of hyaline cartilage. Some preparations of hyaluronidase attack chondroitinsulfuric acids from other sources than the above-mentioned, even when the enzyme has been highly purified (55). On the other hand, hyaluronidase from leech extracts does not disintegrate the chondroitinsulfuric acids mentioned before. Thus the question is left open whether one or two enzymes are involved in these cases. The evidence points to the latter (91).

Other substances related to hyaluronic acid, as, for instance, heparin and the mucins from the respiratory, digestive, and female genital tract, do not act as substrates for hyaluronidase. Several of these substances seem to have some affinity for the enzyme. They seem to combine with the hyaluronidase molecule, inhibiting by competition the degradation of hyaluronic acid (78,81). After removal of the sulfuric acid, this inhibitory power disappears.

When the complexity of hyaluronidase is considered, it is easy to understand that the pH optima of the preparations from different sources vary a great deal. On measuring the formation of reducing substances, two optima, pH 4.5 and 5.7, are found. There is also a shift of the pH optimum from 5.0 to 6.8 with decreasing concentration of the buffer solution (91).

The activity of hyaluronidase is enhanced by salts, especially by sodium chloride (83). The optimal concentration ranges from 0.07 to 0.17 *M*.

When mucopolysaccharides are bound to proteins, they are less easily depolymerized than the corresponding protein-free compound (91).

Hyaluronidase is inhibited by mucopolysaccharides, as mentioned above, and by specific antibodies. Antinvasin I of the sera of different species destroys this enzyme (see page 485).

Biological, physicochemical, and chemical methods are employed in the quantitative determination of hyaluronidase.

A *biological* method often used is the spreading reaction. Hyaluronidase preparations are injected intracutaneously to animals, together with India ink or other dyestuffs. The colored area is compared with the one produced by an injection without the spreading factor. The most accurate method has been developed by Humphrey (63), who measured the increase of areas of the blebs twenty minutes after the injection. The minimal diffusing dose is taken as the least amount of hyaluronidase which will produce a 20% increase in mean area of the blebs, as compared with the mean of the controls. Generally the spreading reaction cannot be considered an accurate assay of hyaluronidase; agreement with results of other methods is often poor (91).

Physicochemical methods are most extensively used. The mucin clot prevention method of McClean and Hale (see preceding section) is suitable for small amounts of hyaluronidase (79). The determination of the drop in viscosity is simple, but rather cumbersome and not well suited for serial determinations. The fundamental principles of this reaction have lately been thoroughly investigated (123). As a result, a method of assay has been devised which depends upon the fact that, within certain prescribed limits, the fall in flow time through an Ostwald viscosimeter of a buffered hyaluronate-hyaluronidase mixture incubated for a fixed time is proportional to the logarithm of the concentration of the enzyme.

Pure hyaluronic acid forms stable colloidal suspensions with diluted serum at pH 4.7, while depolymerized molecules do not show this reaction (65a). This property of hyaluronic acid can be used for accurate and serial determinations. Only small amounts of substrate are required for this procedure and its results are in good agreement with viscosimetric data (91).

The production of reducing groups and *N*-acetyl-hexosamine during the degradation of hyaluronic acid can be measured in the usual way. Thus several methods have been introduced for the *chemical* determination of hyaluronidase.

TABLE VI. HYALURONIDASE OF SNAKE VENOMS

Species	Method	Quantitative data	Ref. Nos.	Notes
Colubrids				
<i>Acanthophis antarcticus</i>	Spreading	14.5 cm. ² (7.2 cm. ²) ^a	33	This venom has a higher spreading power than other preparations with the same viscosity-lowering capacity (23).
<i>Denisonia superba</i>	Spreading	20.0 cm. ² (6.7 cm. ²) ^a	33	
	Spreading	+	23	
	Viscosity lowering	21 units ^b	23	
<i>Elaps fulvius</i>	Spreading	18.6 cm. ² (8.0 cm. ²) ^a	33	This venom has a higher spreading power than other preparations with the same viscosity-lowering capacity (23).
<i>Naia naia</i>	Spreading	10.5 cm. ² (8.0 cm. ²) ^a		
<i>Naia haie</i>	Spreading	+	124	
<i>Notechis scutatus</i>	Spreading	Trace	33	
<i>Notechis scutatus niger</i>	Spreading	+	23	
	Viscosity lowering	111 units ^b	23	
Viperids				
<i>Agkistrodon piscivorus</i>	Spreading	20.0 cm. ² (7.0 cm. ²) ^a	33	Venom contains β -glucosaminase (36). Diffusing factor is accompanied by another substance capable of causing a slow spread of indicator through skin tissue (81).
<i>Bothrops alternatus</i>	Spreading	+	124	
	Viscosity lowering	+	42	
<i>Bothrops jararaca</i>	Spreading	+	124	
	Viscosity lowering	+	42	
<i>Crotalus adamanteus</i>	Spreading	35 cm. ² (7.0 cm. ²) ^a	33	
<i>Crotalus cinereus (atrox)</i>	Spreading	30 cm. ² (3.2 cm. ²) ^a	33	
<i>Crotalus terrificus</i>	Viscosity lowering	+	36,82	
	Spreading	+	124	
	Viscosity lowering	+	42	
	Viscosity lowering	+	81	
<i>Crotalus terrificus</i>	Spreading	30 cm. ² (6.0 cm. ²) ^a	33	
<i>Crotalus durissus</i>				
<i>Echis carinatus</i>	Viscosity lowering	+	42	
<i>Vipera ammodytes</i>	Viscosity lowering	+	42	
<i>Vipera aspis</i>	Spreading	1:90,000 ^c	124	
	Viscosity lowering	+	42	
<i>Vipera russelli</i>	Spreading	+	79	
	Viscosity lowering	+	79	
	Prod. of reduc. subst.	+	79	
	Prod. of N-acetyl glucosamine	+	79	

^a Spreading area of India ink. Figures in parentheses refer to the control. Dilution of venom 1:1000. Reading after 24 hours. ^b Viscosity-reducing units. ^c Spreading activity still positive with given dilution.

D. THE HYALURONIDASE OF SNAKE VENOMS

The results of hyaluronidase determinations of snake venoms are shown in Table VI. It may be seen from this table that the spreading factor and hyaluronidase, the latter being measured by its viscosity-lowering property, have been found repeatedly in the same venom. Taking into consideration all the results obtained, it may be concluded that the spreading factor in snake venoms hitherto investigated is identical with hyaluronidase.

The venom of *Vipera russelli* contains a hyaluronidase which has a pH optimum of 4.6. The activity drops sharply outside the range pH 4.6 to 6.5 (79). This venom does not attack heparin. The hyaluronidase of *Crotalus cinereus* is more sensitive to heat than the lecithinase and proteinase of this venom (82). Destruction of hyaluronidase by formol parallels the disappearance of the toxicity of the venoms of *Vipera* and *Bothrops* (43).

A sixfold concentration of the hyaluronidase of the venom of *Crotalus cinereus* (82) could be obtained by precipitation of the enzyme with half-saturated ammonium sulfate. Different adsorbents absorb either the hyaluronidase or the impurities, so that adsorption methods might obviously be employed for the purification of snake venom hyaluronidase.

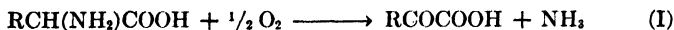
Investigations of several enzymes led to the assumption that in addition to hyaluronidase other diffusing principles are present in snake venoms (23,81). β -Glucosaminase, which is present in the venom of *Crotalus cinereus*, has no viscosity-lowering activity (36).

IV. L-Ophioamino Acid Oxidase

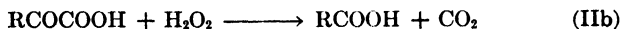
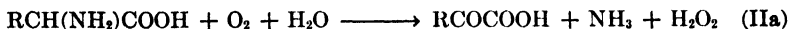
In 1944 we reported (136,150) that an L-amino acid oxidase was present in *Vipera aspis* venom. It is the only nonhydrolytic enzyme to date found in snake venoms. These observations were extended in our laboratory (148,151,154) to the venoms of many other species. The new enzyme, which merits special attention because of its activity and ready solubility, proved to be a very convenient material for the examination of the specificity and reaction mechanism of the enzymic degradation of natural amino acids.

The interaction between the enzyme and its substrates follows different pathways in the presence and absence of catalase. This is illustrated by the following equations (150):

In the presence of catalase:



In the absence of catalase:



The α -keto acid produced in the course of reaction IIa is oxidized by hydrogen peroxide, as was found with pyruvic acid, formed by the action of purified D-amino acid oxidase on D-alanine (100). According to the equations, the activity of the enzyme can be measured by the following procedures: (1)

oxygen consumption—manometric methods; (2) ammonia production—Conway's method and similar procedures; (3) production of keto acid (in the presence of catalase)—the 3,5-dinitrophenylhydrazone of the keto acid yields a reddish-brown color with alkali (103); (4) production of hydrogen peroxide—the peroxide oxidizes hydrogen iodide (102) and phenolphthalin (117), yielding iodine and phenolphthalein. The first two have been adopted as routine procedures, the last method giving only semiquantitative results.

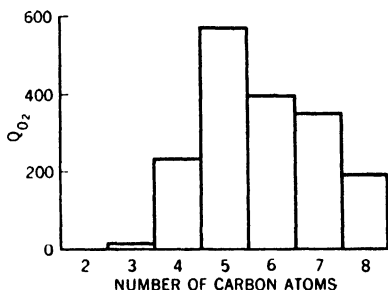


Fig. 2. Oxidation of amino acids with unbranched chains by *Bothrops jararaca* venom.

Optimal conditions for the reaction between enzyme and substrate can be obtained in the following way: 0.25 to 2 mg. dried venom, *M*/15 phosphate buffer pH 7.2, temperature 38°C., L-leucine 0.007 *M* (final concentration). The results are expressed by the symbol QO₂: microliters oxygen per hour per milligram venom.

A. SPECIFICITY

Monoaminomonocarboxylic Acids. With the exception of glycine and D,L-threonine all monoaminomonocarboxylic acids so far examined are oxidized in the presence of the amino acid oxidase (150,151), some with very low velocity (e.g., L-alanine and D,L-serine, 151), some with very high velocity (e.g., L-leucine, L-methionine, and L-phenylalanine, 150). In the unbranched ali-

phatic series the maximal velocity is reached with norvaline (Fig. 2) (139).

TABLE VII
ENZYMIC OXIDATION OF α -AMINOCAPROIC ACIDS BY PURIFIED VENOM
OF *Bitis gabonica*

Amino acid	Qo ₂
Isoleucine.....	158
Leucine.....	584
Norleucine.....	284

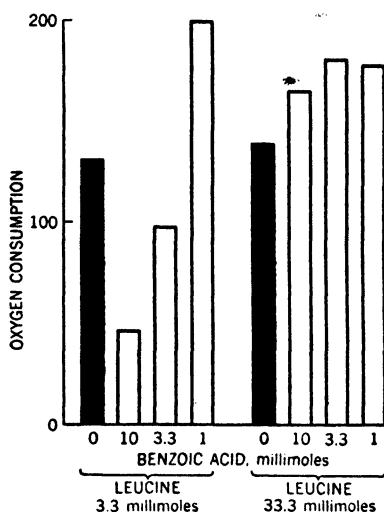


Fig. 3. Competitive inhibition by benzoic acid of the oxidation of leucine by *Vipera aspis* venom. Black, benzoic acid absent; white, acid present. Ordinate, μ l./hr.

It can therefore be concluded that the configuration of the aliphatic chain exerts a definitive influence on the interaction between enzyme and substrate. This can further be demonstrated by comparing the reaction velocities of three different α -aminocaproic acids (Table VII) (139).

L-Diaminomonocarboxylic Acids.

L-Histidine and L-arginine are oxidized with low velocity by the amino acid oxidase, while L-ornithine and L-lysine hardly react at all with the enzyme. However, when the terminal amino group is converted to an acid amide group (L-ornithine to L-citrulline, L-lysine to ϵ -L-benzoyllysine), a degradation takes place. Citrulline is deaminated with high velocity, and

ϵ -L-benzoyllysine with moderate velocity (Table VIII) (151).

Monoaminodicarboxylic Acids. Similar results are obtained with the monoaminodicarboxylic acids. Although no degradation of L-aspartic and glutamic acids is observed, asparagine and glutamine, on the other hand, can be easily oxidized (Table VIII). Again the five-membered chain is more susceptible to enzymic attack than the four membered compound (151).

In Table VIII are listed the substrates of the amino acid oxidase of *Vipera aspis*. Several of these amino acids have never before been attacked enzymically or oxidatively deaminated *in vitro*.

In all cases in which the *L*- α -amino group is substituted no oxidation takes place. Thus proline, hydroxyproline, *N*-methylleucine, *N*-methylphenylalanine (154), and all glycine, leucine, tyrosine, and tryptophan peptides (150) investigated are not attacked. This property of the peptides is applied in the quantitative determination of *L*-peptidases with the aid of snake venoms (110,137-139,143,152).

TABLE VIII
OXIDATION OF AMINO ACIDS BY THE VENOM OF *Vipera aspis*

$Q_{O_2} < 100$	$Q_{O_2} 100-300$	$Q_{O_2} > 300$
L-Alanine	3,5-Diodo-L-tyrosine	L-Methionine
D,L-Serine	3,5-Dibromo-L-tyrosine	L-Leucine
L-Cysteine	Nitro-L-tyrosine	L-Phenylalanine
L-Cystine	3,4-L-Dihydroxyphenylalanine (dopa)	L-Tyrosine*
L-Valine	L-Glutamine	L-Tryptophan
L-Histidine	ϵ -Benzoyl-L-lysine	L-Citrulline
L-Arginine	D,L-Aminobutyric acid	D,L-Norvaline
L-Asparagine	D,L-Aminocaprylic acid	D,L-Norleucine
		D,L-Aminoanthanic acid

* Blaschko (personal communication) has recently observed the degradation of *m*-tyrosine by the *L*-ophioamino acid oxidase.

All attempts to oxidize D,L- α -aminobutansulfonic acid, D,L- α -aminopentansulfonic acid, β -alanine, γ -aminobutyric acid, δ -aminovaleric acid (142), L-tyrosine ethyl ester (151), and many D- α -amino acids (150) gave completely negative results. Some of these substances inhibit oxidation of other amino acids (142). The D-amino acids have so little affinity for the enzyme that minimal amounts of L- α -amino acids in preparations of D- α -amino acids can be determined quantitatively by adding snake venoms. By the same procedure L-amino acids are removed from the D-amino acid preparations.

In conclusion we may indicate the requirements of the specificity of the enzyme as follows: the substrate must possess a free carboxyl group, an unsubstituted α -amino group, and an organic radical. A second amino or carboxyl group inhibits a substance otherwise suitable as a substrate for the enzyme.

B. UNION OF THE ENZYME WITH ITS SUBSTRATE

Investigations on the inhibitory action of several groups of substances have contributed to our knowledge not only of the specificity of the enzyme, but also of the mechanism of the action of the enzyme on the substrate. Several carboxylic acids, as benzoic, salicylic, mandelic, and iodoacetic acids, inhibit the oxidation of L-amino acids by the oxidase (151). This reaction is a clear-cut example of competitive inhibition. The inhibition becomes more pronounced as the concentration of substrate decreases and the concentration of added carboxylic acid increases. With low carboxylic acid concentrations and large amounts of L-amino acids, oxidation of the latter shows an even higher velocity than the controls. It can be shown that the dissociated carboxyl group is responsible for this reaction, since the degree of inhibition increases with increasing dissociation constant (142).

Carboxylic acids are not the only acids able to interfere with substrates of the L-amino acid oxidase. Some aliphatic α -aminosulfonic acids, as well as many aromatic sulfonic acids, are powerful inhibitors: *e.g.*, 2,5-dichlorobenzenesulfonic acid, 2,5-dibromobenzenesulfonic acid, azobenzenesulfonic acid, *p*-hydroxyazobenzenesulfonic acid, *o*-chlorotoluenesulfonic acid, 4-nitrotoluene-2-sulfonic acid, and some sulfonamides employed therapeutically (150,151). We have shown for the first time that these substances are able to compete with amino acids for the enzymes concerned in amino acid metabolism (150,151).

Since many acids appear to prevent reaction between the carboxyl group of the substrate and the enzyme, ammonia should be able to interfere in the same manner with the amino group. This is true for the L-amino acid oxidase of rat kidney (12) but not for the enzyme of snake venom (154). On the other hand, the amino group of tyrosine ethyl ester acts as an inhibitor (151).

Although hydrocyanic acid does not exert any influence, at least in the concentrations generally applied, several other carbonyl reagents, as semicarbazide hydrochloride, hydroxylamine hydrochloride, 3,5-dinitrophenylhydrazine, and dimethylcyclohexanedione (dimedon), take part in competitive inhibitory reactions (154). However, the inhibition is not so accentuated as in the case of enzymes like diamine oxidase (133) and histidine carboxylase (129).

The competition of different amino acids for the enzyme seems to prove that the same agent may act as catalyst in the oxidation of all

the amino acids listed in Table VIII (150). Even L-proline, which is not attacked, can interfere with the oxidation of other L-amino acids, exhibiting in this way some affinity for the oxidase (154).

The amino acid oxidase is inhibited by high substrate concentrations (150). This result may be understood by assuming that there are two sites of attachment between enzyme and substrate (56). Thus at high concentrations the possibility arises of the combination of two substrate molecules with one enzyme molecule (for another thoroughly examined example, see 133, Fig. 5). The amino acid may combine at the α -amino and at the dissociated carboxyl group, the enzyme at a carbonyl and a positively charged group.

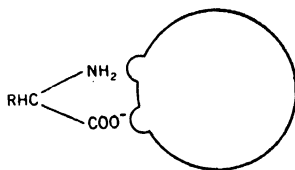


Fig. 4. Tentative model of L-ophioamino acid oxidase.

The experimental results hitherto obtained can be consolidated to form the model shown in Figure 4.

C. DIFFERENCES BETWEEN THE L-OPHIOAMINO ACID OXIDASE AND OTHER L-AMINO ACID OXIDASES

No other known amino acid oxidase shows the same properties as the enzyme from snake venoms. The L-amino acid oxidase of mammalian kidney found by Krebs is very sensitive to dilution and to addition of octyl alcohol; the snake venom oxidase is not influenced by these procedures (70,71). An enzyme investigated by Edlbacher and Grauer (37) and occurring in the kidneys of several species oxidizes L-valine more slowly than L-alanine, but the reverse is true for snake venom oxidase.

Regarding the specificity, a close relationship can be established between the amino acid oxidase of *Proteus vulgaris* and snake venoms (121). Both enzymes fail to attack N-methylated amino acids and L-proline. But in many other respects the differences between the two enzymes are considerable. They are summarized in Table IX. Some of the main differences between the enzyme of the venoms and the oxidase in rat kidney isolated by Green and co-workers (12,13) have already been mentioned; they are summarized in Table X. It was later found that the rat kidney oxidase attacks L- α -hydroxy acids (13,14). These results are corroborated by those obtained earlier in our laboratory (153), which were extended later (64,111).

TABLE IX
SOME PROPERTIES OF THE L-AMINO ACID OXIDASES OF *Proteus vulgaris*
AND OF SNAKE VENOMS

Property	<i>Proteus vulgaris</i> oxidase	Snake venom oxidase
Oxidation of L-valine	—	+
Inhibition by octyl alcohol	+	—
by hydrocyanic acid	+	—
by benzoic acid	—	+
by iodoacetic acid	—	+
Formation of hydrogen peroxide	—	+
Inhibition by high substrate concentrations	—	+

TABLE X
SOME PROPERTIES OF THE L-AMINO ACID OXIDASES OF RAT KIDNEY
AND OF SNAKE VENOMS

Property	Rat kidney oxidase	Snake venom oxidase
Oxidation of proline	+	—
of N-methylated amino acids	+	—
of L- α -hydroxy acids	+	—
Inhibition by ammonium salts	+	—

Thus the enzyme of snake venoms (and snake tissues) appears to be different from all other known L-amino acid oxidases (see 148 for discussion of other L-amino acid oxidases), and has therefore been named L-ophioamino acid oxidase (ophio oxidase).

D. OCCURRENCE OF L-OPHIOAMINO ACID OXIDASE

From the biological point of view it is important to know that L-ophioamino acid oxidase occurs in venoms as well as in several tissues of venomous (*Vipera aspis*) and nonvenomous snakes (*Tropidonotus natrix* and *T. tessellatus*). The enzyme activity of the organs decreases in the following order: lung, liver, kidney, erythrocytes (148). Since the ophio oxidase of these tissues attacks the same pattern of L-amino acids and is inhibited by aromatic sulfonic acids in a similar way as the oxidase of the venoms, it follows that the two enzymes are very closely related to each other or are even identical.

The activity of the L-ophioamino acid oxidase generally attains higher values in the venoms of viperids than in the venoms of colu-

brids. To a certain point, the activity is specific for a genus. Thus, in the group of pit vipers, the genus *Bothrops* exhibits higher activities than the genus *Crotalus*. No trace of ophio oxidase was found in the venoms of the colubrid *Demansia textilis* and the viperid *Bothrops itapetiningae*. These are the only venoms which are pure white; all the others are yellow (140).

TABLE XI
DEGRADATION OF L-LEUCINE BY DIFFERENT SNAKE VENOMS

Species	Qo ₂	Species	Qo ₂
Colubrids		Viperids	
<i>Acanthophis antarcticus</i>	80	<i>Bothrops jararaca</i>	750
<i>Bungarus coeruleus</i>	250	<i>Bothrops jararacussu</i>	400
<i>Bungarus fasciatus</i>	230	<i>Bothrops itapetiningae</i>	0
<i>Demansia textilis</i>	0	<i>Bothrops neuwiedii</i>	590
<i>Dendraspis angusticeps</i>	50	<i>Bothrops nummifera</i>	190
<i>Denisonia superba</i>	60	<i>Cerastes vipera</i>	420
<i>Elaps corallinus</i>	10	<i>Crotalus adamanteus</i>	50
<i>Naia bungarus</i>	380	<i>Crotalus cinereus</i>	100
<i>Naia flava</i>	60	<i>Crotalus horridus</i>	70
<i>Naia haie</i>	250	<i>Crotalus lucasensis</i>	60
<i>Naia melanoleuca</i>	240	<i>Crotalus ruber</i>	10
<i>Naia naia</i>	100	<i>Crotalus terrificus terrificus</i>	10
<i>Naia nigricollis</i>	100	<i>Crotalus terrificus basilicus</i>	80
<i>Notechis scutatus</i>	40	<i>Crotalus viridis</i>	270
<i>Pseudechis australis</i>	50	<i>Crotalus viridis oreganus</i>	250
<i>Pseudechis porphyriacus</i>	190	<i>Echis carinatus</i>	130
<i>Sepedon haemachates</i>	120	<i>Sistrurus catenatus</i>	100
Viperids		<i>Trimeresurus gramineus</i>	620
<i>Agkistrodon mokasen</i>	45	<i>Vipera ammodytes</i>	310
<i>Agkistrodon piscivorus</i>	380	<i>Vipera aspis</i>	610
<i>Bitis arietans</i>	380	<i>Vipera aspis Hugyi</i>	2440
<i>Bitis gabonica</i>	320	<i>Vipera berus</i>	+
<i>Bothrops alternatus</i>	340	<i>Vipera latastei</i>	620
<i>Bothrops atrox</i>	760	<i>Vipera lebetina</i>	1570
<i>Bothrops cotiara</i>	390	<i>Vipera russelli</i>	760

TABLE XII
GEOGRAPHICAL VARIETIES OF SNAKE SPECIES AND THEIR VENOMS

Species	Venom color	Qo ₂ (leucine)
<i>Denisonia superba</i>	Yellow	60
<i>Denisonia superba</i> (variety living at higher altitude)		
<i>Notechis scutatus</i>	Creamish white	10
<i>Notechis scutatus</i>	Slightly yellow	40
<i>Notechis scutatus niger</i>	Creamish white	10
<i>Vipera aspis</i>	Yellow	90
<i>Vipera aspis</i> (Département du Gers)	Yellow	610
	White	3

It is well known that geographical varieties of a single species can produce venoms of different color (127). In three cases investigated the activity of L-ophioamino acid oxidase runs roughly parallel to the intensity of the yellow color (Table XII). Fluorometric assays led to the assumption that riboflavin is present in *V. aspis* and *Naia naia* venoms (21). These results were confirmed and extended by microbiological methods in the writer's laboratory (143,149). Consequently we know that the yellow color of snake venoms is partly due to riboflavin (Table XIII). The venom of *Vipera aspis* showed no evidence of the presence of either thiamine, nicotinic acid, or pantothenic acid. In purification experiments the colored and the active substances always occur in the same fraction (143).

* TABLE XIII
RIBOFLAVIN CONTENT OF SNAKE VENOMS^a

Genus	Milligram, per cent
<i>Naia naia</i>	3.0 to 6.6 ^b
<i>Naia melanoleuca</i>	6.8
<i>Bitis gabonica</i>	7.0
<i>Bothrops atrox</i>	18.0
<i>Vipera aspis</i>	5.5 to 20.0 ^b
<i>Vipera latastei</i>	2.7
<i>Vipera lebetina</i>	16.8

^a White venoms of *V. aspis* (cf. Table XII) and of *Bothrops itapetiningae* are almost or completely devoid of riboflavin. ^b Different samples.

V. Proinvasin I

The intercellular gel of the connective tissue forms a strong barrier against the rapid diffusion of bacteria and venoms. This resistance can be overcome by the aggressive agents with the aid of the hyaluronidase they contain. The invaded organism is able to retain the adequate colloidal state of its connective tissue by inhibiting this enzyme. In addition to the inhibitors mentioned before (see page 471) several others have been investigated. The inhibition of bacterial hyaluronidase by normal human serum was found first shown in 1940 (60). McClean (78) discovered the inhibitory action of guinea pig, rabbit, sheep, horse, mouse, and human serum on hyaluronidase prepared from bull, rabbit, and mouse testes, from streptococci, *Clostridium welchii*, and from viper and scorpion venom. The inhibitory action of the sera was associated with the pseudoglobulin fraction.

Haas (52-54) reported the existence of a hyaluronidase-destroying agent in the serum of man, horse, beef, hog, rabbit, chicken, and carp. This antihyaluronidase, called antinvasin I, inactivates the hyaluronidase of testes (man, bull), and various bacteria, but not that contained in *Agkistrodon piscivorus* venom. The factor is heat labile and does not dialyze through cellophane membranes. Lately this antinvasin has been separated into two factors (49). Each of these fractions is practically inactive in an isolated state, but recombination results in the original antivasin I activity.

The antivasin I is in turn inactivated by several bacteria and venoms. The heat-labile agent responsible for this reaction is called proinvasin I. Thus the venom of *Agkistrodon piscivorus* destroys the antinvasin of human, chicken, hog, and golden carp plasma. The concentrations of proinvasin I and hyaluronidase vary a great deal. This is shown by the data given in Table XIV. The relative proportion of proinvasin I to hyaluronidase may determine the course of invasion of the agents. With large amounts of proinvasin I, such as are present in venom of *Agkistrodon piscivorus*, the destruction of antivasin I takes place so rapidly that the reaction antinvasin \rightarrow hyaluronidase becomes negligible. In such a case hyaluronidase will not be destroyed by antinvasin I, and invasion can proceed. With small amounts of proinvasin I, the destruction of antivasin I becomes unimportant. Under these conditions antinvasin I in plasma would be capable of preventing invasion (53).

TABLE XIV
RELATIVE PROPORTION OF PROINVASIN I TO HYALURONIDASE IN SNAKE VENOM

Source of enzyme	Milligrams required for		Proinvasin I/ hyaluronidase
	Hyaluronidase action ^a	Proinvasin I action ^b	
<i>Agkistrodon piscivorus</i>	0.10	0.009	11.0
<i>Crotalus adamanteus</i>	0.06	0.05	1.2

^a Concentration of hyaluronidase which will depolymerize 50% of the polysaccharide in ten minutes at 25°.

^b Concentration of proinvasin which will cause destruction of 50% antivasin I in ten minutes at 25°.

The proinvasin I of snake venom (*Agkistrodon piscivorus*) may be destroyed by the action of an agent called antinvasin II, which differs

from antinvasin I and is more heat stable than the latter. Its presence has been shown in human, beef, chicken, and carp plasma.

Only few data are available on the properties and enzyme nature of proinvasin I and antinvasin II; nothing is known concerning interaction of antinvasin I or II and proinvasin I. Although the mechanism of these reactions is not yet understood, they nevertheless illustrate the profound mutual influence of different components of a venom on their respective functions.

VI. Biological Significance of Snake Venom Enzymes

Before discussing the role of single enzymes we must pause to consider the biological significance of the venoms as a whole. From the anatomical, physiological, and biochemical findings, it would appear that the venom-producing glands originally had a digestive function. The more primitive genera, *Colubridae opisthoglyphae*, have developed a simple fang structure in the rear of the upper jaw and a comparatively mild poison. These reptiles, with one exception (*Despholidus typus*, 50), have never been included among the dangerous serpents. They swallow their prey, injecting venoms by which the digestive process is initiated. Unlike other animals the serpents cannot mix the secretion products of their digestive glands with their food by chewing it or tearing it to pieces. Instead they use their highly developed injection apparatus to apply digestive agents to the prey.

Apart from their digestive role the highly concentrated and powerful enzymes can exert a strong poisonous effect. This may be demonstrated by an enzyme of *plant* origin: 0.15 mg. crystalline urease, injected intravenously, is sufficient to kill a rabbit. This toxic effect is due to the ammonia produced by the action of this enzyme on urea. Chickens are immune to urease, since their blood has a very low urea level (122).

In the course of phylogenetic development, the length of the maxilla decreased. Owing to this process the position of the fangs gradually shifted toward the front of the mouth. They thus developed into weapons of defense and attack (*Colubridae proteroglyphae*). In the *Viperidae* family the fangs were even further specialized in this direction.

Presence in snake venoms of enzymes like proteases, peptidases, phosphatases, esterases, and lecithinases agrees with these assump-

tions. All the enzymes mentioned in the foregoing discussion except L-ophioamino acid oxidase and hyaluronidase are encountered in the digestive juices of other animals. Some of the poison enzymes may be digestive agents; some others may have been developed in the direction of highly toxic substances, and some display activities in both directions.

The poisons are not able to fulfil their physiological role before they are spread rapidly from the point of inoculation throughout the whole body of the prey. But serious obstacles are raised against such a propagation. In a previous section it was shown that the spaces between the cells and the fibers of the connective tissue of the skin are occupied by a hyaluronic acid gel. Since many components of the poisons are of colloidal nature, their diffusion velocity in such a jelly must be extremely low. The venoms overcome this barrier by hydrolyzing the hyaluronic acid with the aid of hyaluronidase and thus immediately reduce to very low values the viscosity of the connective tissue. Since the body of the prey displays hyaluronidase-destroying activity (antivasin I), the snake enzyme must be protected by proinvasin I.

Venoms, especially those from viperid snakes, produce some other local reactions, in addition to the spreading effect described above. The increase of permeability of the capillary walls is not to be ascribed to the direct action of hyaluronidase, since the capillary cement does not contain hyaluronic acid. However, the enzyme contributes indirectly to the lesions of the capillaries by destroying surrounding ground substance, which supports the vessels. Lecithinases A increase this effect by attacking the lipid layer of the endothelial cell surfaces (28). As a result lysolecithins are formed which give an expanded, fragile, and more permeable film (62). The powerful proteinases contained in many snake poisons lead to actual dissolution of blood vessels and subsequent extravasation of erythrocytes and serum into the tissues. A widespread hemorrhagic edema is the result. In several instances it has been noticed that the "spreading" reaction produced by snake venom was greater than could be accounted for by the hyaluronidase content (Table VI, page 475); these observations are easily explained by the presence of the lecithinases and proteinases. The combined effect of these enzymes can be demonstrated by injecting small doses of a poison into the subcutis of mice. Within a short time the animal becomes anemic and severe hemor-

rhagic edema spreads not only over the subcutaneous tissue of the whole back, but extends also to the belly.

When men or larger animals are bitten by big viperid snakes and happen not to succumb, heavy necrosis often occurs at the site of the bite. This effect obviously is not to be attributed to the immobilizing and killing action, but to the primitive digestive properties of snake poisons.

Absorption appears to occur mainly from the region of the advancing edge of the edema. The venom-containing edematous fluid seems to be absorbed by the lymphatics and to pass up the corresponding chain of nodes. These become greatly swollen and hemorrhagic and show marked hyperplasia of the reticuloendothelial cells (46).

Here we may discuss the problem of the physiological function of L-ophioamino acid oxidase. Unlike most of the other venom enzymes, L-ophioamino acid oxidase was discovered in enzyme rather than in biological studies. We must therefore try to find out its role in the mechanism of the poisonous action. Despite the fact that this enzyme is very common (see page 483), a few venoms nevertheless are devoid of it. It is therefore not absolutely essential to the deleterious effect of snake bites.

Comparatively large amounts of L-ophioamino acid oxidase are present in specimens of the genera *Bothrops* and *Vipera*—probably 1–3% of the crude dry product. These venoms may serve to verify whether the presence of this enzyme is necessary for their poisonous action. We therefore neutralized the poisons of *Bothrops jararaca*, *Vipera aspis*, and *Crotalus terrificus* with the corresponding antisera. Even after the addition of a great surplus of antiserum to the venom, not the slightest inhibition of L-ophioamino acid oxidase was observed (143). From these results we may conclude that this enzyme is a nontoxic component.

It has been known for many years that animals killed by snake bites undergo autolysis and putrefaction at a higher rate than usual, even when the corresponding venoms do not display high proteolytic activity. In agreement with these observations it could be shown that venoms are capable of activating proteases (80) and peptidases (125). The mechanism of these reactions was not elucidated. Studying the influence of many venoms on peptidases of different tissues, we were able to confirm and extend the work of previous authors. The results point to L-ophioamino acid oxidase as the component responsi-

ble for this activating power of the poisons. The white venoms, which lack this enzyme (*Vipera aspis*, *Demansia textilis*, and *Bothrops itapetiningae*), do not in any way influence the peptide-splitting effect of tissue extract (143).

A possible interpretation of these results is that the reaction products of the system peptide-peptidase are removed by converting them to α -keto acids. As a consequence the equilibrium is disturbed and can be restored only by the degradation of more peptide molecules. It has been shown by Bergmann and co-workers (9) that this equilibrium is not shifted so far toward hydrolysis that enzymic synthesis could not take place at an easily measurable rate.

In complete accord with these results are some observations of certain French investigators on the white venom of *Vipera aspis* from southern France (Département du Gers). Although the yellow venom of this species from other parts of France causes necrosis at the site of inoculation, the white poison does not (22).

The protease-enhancing effect of L-ophioamino acid oxidase can take place in all living cells, since they all contain proteases and peptidases susceptible to activation (ophio oxidase is apparently not responsible for the inactivation of serum hypertensin by snake venoms, 26).^{*} Thus, a preformed chain of reactions is started in the organism of the prey. Similar mechanisms have been encountered in the course of the studies on snake venom effects (see below).

In summing up all known facts we may conclude that L-ophioamino acid oxidase is a digestive rather than a toxic agent which activates proteolytic enzymes. Ophio oxidase is so far the only enzyme of the digestive tract that is not a hydrolase. Quite possibly, similar mechanisms may be found one day to occur in the metabolism of higher animals.

In addition to the local disturbances, general effects extending to many organs may be expected as soon as the poison invades the blood. The circulatory system itself is affected in more than one way. Many venoms produce heavy thrombosis in larger vessels as, for instance, in the vena portae (73). At the site of the venom inoculation thrombosis also occurs in smaller vessels exposed to the direct attack of the poison (46).

^{*} A mouse killed by the action of viper venom (*Vipera aspis*), which is nearly devoid of proteolytic enzymes, is digested by the snake in about three days; but when the snake is prevented from using its fangs the process lasts for five to eight days (personal communication from C. Stemmler-Morath).

The powerful proteinases present in many snake venoms act in several ways on the clotting mechanism of the blood. They prevent clotting by dissolving the prothrombin and/or the fibrinogen. The formation of thrombin from prothrombin and the conversion of fibrinogen to a fibrillar gel probably are also due to their action. Neither thrombin nor calcium ions are necessary for the latter process (34). In the venom of *daboia*, which has a remarkably high clotting power, a mechanism different from all others hitherto mentioned is responsible for the clotting reaction (38).

It has been claimed that enzyme reactions are involved in the production of shock. The sharp decrease of blood pressure was ascribed to histamine, since perfused organs liberate histamine when venoms are added to the perfusion fluid. A close parallel was established between hemolytic power and capacity to liberate histamine (44,45,66, 69). The lysolecithin formed by lecithinase causes cell injury and thus the subsequent release of histamine and coagulable proteins. Crystals of the so-called neurotoxin from *Crotalus terrificus*, which show the same ratio of toxicity to lecithinase A as the crude venom, are highly toxic (118). On the other hand, a syndrome very similar to anaphylactic shock could be produced by injection of a solution prepared from crystalline trypsin. 20 mg. killed normal guinea pigs in two minutes (109). See note appended to reference (155).

Many lesions of brain and nerves are caused by various snake venoms. The attack of "neurotoxins" is directed toward different parts of the nervous system. There are good reasons for assuming that enzyme processes are involved in the action of neurotoxins. Unfortunately only few exact data are available and no clear-cut relationships have as yet been established. The difficulties are considerably enhanced by the term neurotoxin, which is used by different authors in a greatly different sense. (See note appended to reference (155).

A common cause of death in mammals poisoned by colubrid venoms is asphyxia resulting from failure of respiratory movements (for literature, see Kellaway, 67). This effect is due to the so-called curare-like action, found only in colubrid venoms (until recently, when it was reported that the venom of *Trimeresurus mucrosquamatus*—Habu from Korea—a species of viperid, also exerts a curare-like action, 99). The effect was supposed to be related to ChE (65). The acetylcholine produced at the motor end plates was believed to be destroyed by the extremely active ChE before it could perform its

function as transmitter of nerve impulses. This hypothesis received further support from the fact that the enzyme could be found only in colubrid venoms (see page 469). However, the "neurotoxin" and the ChE could be separated into two fractions. The former displayed no ChE activity and the latter no curare-like action (47,113). The two agents differ also in other respects, as for instance in heat lability (47). Diisopropyl fluorophosphate (DFP), which inhibits ChE *in vivo*, does not protect animals against the fatal effect of cobra venom (18).

The venoms are capable of penetrating from the blood into other tissues (intestinal mucosa, motor end plates in muscles, etc.). Probably the same agents are responsible for this process as well as for the invasion of the blood. For instance, it has been shown recently by histological methods that cobra venom overcomes the blood-brain barrier (20).

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